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THE STUDY OF THE OXIDATION OF α-KETOGLUTARATE AND THE COUPLED PHOSPHORYLATION BY THE TISSUE HOMOGENATES OF NORMAL AND THIAMINE-DEFICIENT RATS.

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In view of the importance of α-ketoglutarate as an intermediate in the metabolism of carbohydrate, fat and amino acids, its oxidation and the coupled phosphorylation in animal tissues have been studied by several investigators (*1–6*). Shimizu in this laboratory found that the concentration of α-ketoglutarate in human blood was lowered by the injection of thiamine (*7*), while the amounts of α-ketoglutarate were increased remarkably in blood and urine of the thiamine-deficient rats (*8*). Thus it became necessary to us to investigate the rate of the oxidation of α-ketoglutarate by the thiamine-deficient tissues. In the literature there are only few reports concerned with this problem. Barron (*9*) showed, using the tissue slice technique, that the rate of oxygen consumption by the tissues from thiamine-deficient rats in the presence of α-ketoglutrate was decreased below normal, and the addition of thiamine restored the rate of oxygen uptake to some extent. Since 1945 Potter and others (*10–14*) demonstrated that the tissue homogenates were capable of coupling the energy of the oxidation of C_4-dicarboxylic and α-ketoglutaric acids with the phosphorylation, and that the uptake of phosphate during the short periods of the incubation was proportional to the amounts of the tissue employed. But we cannot find any information on the rate of the formation of high-energy phosphate bonds coupled with the oxidation of α-ketoglutarate in the thiamine-deficient tissues.

In the present study we employed the homogenate system of Potter and others, and examined the oxidation of α-ketoglutarate and the coupled phosphorylation in the tissue homogenates of normal, or thiamine-deficient, or pair-fed rats. And thus we tried to clarify the relationship between thiamine or diphosphothiamine and α-ketoglutarate metabolism involving the oxidative phosphorylation.

EXPERIMENTAL

Treatment of Animals—Rats weighing about 60 g. were divided into three groups, the first of which received a thiamine-free synthetic ration. The second, a control group, received the nutritionally complete synthetic ration *ad libitum*, and the third, pair-fed control group, was given the same ration as the first group but with the thiamine added. All of the three groups received sufficient quantities of the components of the vitamin B_2 group (riboflavin, niacine, pyridoxine, pantothenate and choline) for the complete nutrition. After 4–5 weeks, when the deficient group showed remarkable deficiency-symptoms, all the rats of the three groups were killed and their tissues were used for the respiration experiments.

The Basic System for the Determination of Respiration—The basic system employed was 50 mg. of tissue homogenate (liver, kidney, or heart) placed in a reaction mixture containing α-ketoglutarate, inorganic phosphate, adenosine triphosphate (ATP), and Mg^{++}. In the experiment of the oxidation of α-ketoglutarate we usually added a high concentration of potassium malonate to block the action of tissue succinic dehydrogenase. In order to investigate the oxidative phosphorylation we added creatine as a phosphate acceptor and fluoride as an inhibitor of tissue ATP-ase.

Analytical—The rate of the oxygen uptake was measured at 37° by means of a conventional Warburg apparatus. The "true" inorganic phosphate was measured by Lowry-Lopez method (*15*). The reaction in each vessel was stopped by the addition of a cold 10% solution of trichloroacetic acid, and the precipitated protein was filtered off under low temperature, and an aliquot of the filtrate was used to estimate the contents of inorganic phosphate. The total nitrogen of the homogenates was determined by the micro-Kjeldahl method, and the value of $Q_{O_2}(N)$ was computed for the purpose of determining the relative rate of oxygen consumption of each tissue homogenate. The thiamine content was estimated by the thiochrome method after Taka-enzyme had been added to a sample of tissue and permitted to incubate overnight at pH 4.5. The amount of α-ketoglutarate was determined by Clift-Cook's method for estimating BBS (*16*) or by Shimizu's for estimating α-ketoglutarate (*17*).

Tissue Preparation—Rat kidney, liver and heart homogenates were used in this experiment. Rats were killed by decapitation and the tissues used were weighed and placed in homogenizer tubes. The

Potter and Elvehjem glass homogenizer was used. The homogenization was carried out under low temperature, and 5 volumes of ice cold KCl-Na_2HPO_4 solution (the mixture of 0.9% KCl and 0.1 *M* Na_2HPO_4 in the proportion of 10:1) were added to 1 volume of the homogenate. 0.3 ml. of this homogenate was put into each Warburg flask. The above amount of Na_2HPO_4 was sufficient to neutralize the acid formed when the cells were ruptured during the homogenization procedure.

Preparation of Reaction Components—α-Ketoglutarate used as the substrate was synthesized by the procedure tested and proved by Fuson and Marvell (*18*). ATP was isolated from rabbit muscle in the form of barium salt by the method of Dounce and others (*19*). The barium salt was made fit for use by dissolving it in 1.0 *N* sulfuric acid to precipitate the barium as sulfate, and then by neutralizing the solution with KOH. DPN was prepared from baker's yeast by the method of Williamson and Green (*20*). Cytochrome c was prepared from beef hearts by the method of Keilin and Hartree (*21*). In our experiment the addition of either cytochrome c or DPN showed hardly any effect upon either the rate of oxidation of α-ketoglutarate or the coupled phosphorylation. Consequently we omitted these compounds from our basic system.

Reaction Mixture—In the experiment of the oxidation each manometer vessel contained 0.5 ml. of *M*/50 Na-α-ketoglutarate, 0.5 ml. of *M*/200 ATP, 0.4 ml. of *M*/10 K-phosphate buffer (pH 7.4), 0.4 ml. of 1/2 *M* KCl, 0.1 ml. of 1 *M* $MgCl_2$, and 0.3 ml. of 0.3 *M* K-malonate to which enough redistilled water was added to make a total volume of 3 ml. Just before the measurement of the oxygen uptake 0.3 ml. of the 16.7% freshly prepared homogenate was added. As Potter had pointed out already (*22*), when malonate was added in such a relatively high concentration to the reaction mixture, the addition of a high concentration of Mg^{++} ion was absolutely necessary. The concentration of K-malonate used was proved by preliminary experiments to be sufficient to inhibit the succinic dehydrogenase of the tissue homogenate.

The reaction mixture for the study of the oxidative phosphorylation consisted of the following components, which were added in the order given: 30 mg. of dry creatine hydrate, water to make a total volume of 3.0 ml., 0.2 ml. of 0.5 *M* KCl, 0.2 ml. of 0.1 *M* $MgCl_2$, 0.2 ml. of 0.1 *M* K-phosphate buffer (pH 7.4), 0.5 ml. of *M*/200 K-ATP, 0.5 ml. of *M*/50 Na-α-ketoglutarate, and finally 0.2 ml. of an adequate concentration of NaF.

Measurement of Oxygen Consumption—Each center cup of the War-

burg vessel contained 0.2 ml. of 2 *N* KOH and a folded filter paper. The gas phase was air. The incubation was carried out by shaking at 37°, and the stopcocks were closed after a 10 minutes equilibration period.

On estimating the rate of α-ketoglutarate oxidation, the oxygen consumption in 10 minutes of equilibration period was omitted, and the oxygen consumption during 60 minutes incubation period was calculated as Q_{O_2} (N) $_{\alpha\text{-ketoglutarate}}$ ($\mu l.$ O_2 taken up per mg. tissue nitrogen per hour, corrected for the endogenous O_2-uptake).

In order to estimate the oxidative phosphorylation, the oxygen consumption was corrected so as to include the uptake during the equilibration, on the assumption that the reaction proceeded at the same rate during this period as during the next 10 minutes period, and this oxygen consumption was used to calculate the P:O ratio.

RESULTS

I. *The Oxidation of α-Ketoglutarate by the Homogenized Liver and Kidney of Thamine-Deficient Rats and Normal or Pair-Fed Controls.*

Components of the System—The effect of the addition of cytochrome c and DPN was not great in our enzyme system, as shown in Table I. Consequently we omitted the addition of these components in the

TABLE I

The Influence of Cytochrome c and DPN on the Oxidation of α-Ketoglutarate of Rat Liver Homogenates in the Presence of ATP (Malonate Added)

0.1 % Cytochrome c *ml.*	0.1	——	0.1	——
0.01 *M* DPN *ml.*	0.1	0.1	——	——
Oxygen consumption, $\mu l.$ (60 minutes)*	92.6	84.2	80.6	80.7

The Warburg vessels contained 0.5 ml. of 0.02 *M* Na-α-ketoglutarate, 0.5 ml. of 0.005 *M* K-ATP, 0.4 ml. of 0.1 *M* K-phosphate buffer (pH 7.4), 0.1 ml. of 1 *M* $MgCl_2$, 0.3 ml. of 0.3 *M* K-malonate, 0.4 ml. of 0.5 *M* KCl and 0.3 ml. of 16.7% of the homogenate in a total volume of 3 ml., made up with water. Gas phase: air. The taps were closed after a 10-minute equilibration period at 37°, and then the incubation continued for next one hour.

* The oxygen consumption for the endogenous respiration was deducted from the total oxygen consumption, and the values are shown here.

following experiments. The addition of ATP, however, increased the oxygen comsumption of the enzyme system remarkably, as shown in Table II.

TABLE II

The Influence of K-ATP on the Oxidation of α-Ketoglutarate of Rat Heart Homogenates

Addition		Rate of oxygen uptake (50 minutes incubation)
α-Ketoglutarate	ATP	μl.
+	+	82.6
−	+	34.8
+	−	36.1
−	−	29.8

The experimental conditions were the same as in Table I.

Thiamine in the Homogenates and the Oxidation of α-Ketoglutarate—Table III represents the results of the oxidation of α-ketoglutarate by the homogenates of kidney and liver from thiamine-deficient rats, pair-fed controls and controls fed *ad libitum*.

The values in the table are all corrected for the blank experiments without the addition of α-ketoglutarate. The endogenous oxygen consumption of the thiamine-deficient homogenates of liver and kidney was not significantly lower than that of the control homogenates. On the other hand, the oxygen consumption by the kidney or liver homogenates of thiamine-deficient rats in the presence of α-ketoglutarate was distinctly less than that of the pair-fed controls and the controls fed *ad libitum*. $Q_{O_2}(N)_{\alpha\text{-ketoglutarate}}$ of the liver homogenates of thiamine-deficient rats was decreased in proportion to the decrease of the thiamine content of the tissue as shown in Table III. This experiment shows that the oxidation of α-ketoglutarate of tissues is related to their thiamine contents.

Table IV shows the effect on the oxidation of α-ketoglutarate of the addition of thiamine and diphosphothiamine to the homogenates of the livers of thiamine-deficient rats in the presence of malonate. The use of catalytic amounts of both thiamine and diphosphothiamine together restored somewhat in every case the oxygen uptake of the

TABLE III

The Relation of $Qo_2(N)_{\alpha\text{-Ketoglutarate}}$ to the Thiamine Content of the Rat Kidney and Liver Homogenate.

Group of rats	Rat No.	$Qo_2(N)_{\alpha\text{-ketoglutarate}}$		Thaiamine content of liver. *μg. per cent*
		Kidney	Liver	
Thiamine-deficient	1	8.4	4.3	74
	2		16.4	165
	6	14.3	4.1	45
	9	14.4	5.9	
	10		2.3	25
	17		2.8	44
	18		5.8	44
	22		11.4	
	23	9.9	0.8	
	24		6.2	68
	27		5.1	56
	28		5.9	55
Pair-fed controls	7	30.7	37.5	1143
	11	21.7	9.3	
	20		14.2	352
	25		45.5	1248
	30		45.6	1127
Normal	4	70.5	25.1	1345
	8	37.2	44.8	
	12	62.2	51.2	
	21	91.0	41.0	621
	26	54.0	31.8	

The experimental conditions were the same as in Table I.

homogenates, and in some cases it brought the level of the $Qo_2(N)_{\alpha\text{-ketoglutarate}}$ to that of the pair-fed controls. These data in the table support the view that diphosphothiamine plays a part in the oxidation of α-ketoglutarate in the enzyme system of the homogenates. By comparison of the values of the disappearance of α-ketoglutarate with those of the oxygen consumption it was confirmed that the oxidation product of α-ketoglutarate was succinic acid.

TABLE IV

The Effect upon the Oxidation of α-Ketoglutarate by the Addition of Diphosphothiamine and Thiamine to the Homogenates of Thiamine-Deficient Rat Liver

Substance added		No addition	Thiamine (10 μg.) + diphosphothiamine (1 μg.)
Group of rats	Rat No.	$Q_{O_2}(N)$ α-ketoglutarate	$Q_{O_2}(N)$ α-ketoglutarate
Normal	12	51.2	53.2
	8	44.8	47.3
	21	41.0	
Pair-fed controls	11	9.3	9.6
	7	37.5	
	20	29.5	29.5
Thiamine-deficient	18	5.8	8.3
	17	2.7	8.0
	10	2.3	6.7
	23	0.8	4.5
	27	5.1	6.8
	24	6.2	15.2
	28	5.9	9.6

The experimental conditions were the same as in Table I.

II. *The Phosphorylation Coupled with the Oxidation of α-Ketoglutarate in the Homogenized Heart and Kidney of Thiamine-Deficient Rats and Normal or Pair-Fed Controls.*

Components of the System—The oxidative phosphorylation was observed over a period of 20 minutes in the above homogenate system, which lacked malonate, and which acted on α-ketoglutarate as oxidizable substrate, and on creatine as phosphate acceptor in the presence of NaF. The concentration of inorganic phosphate was proved to be lowered, indicating that the phosphorylation of creatine coupled with the oxidation of α-ketoglutarate occurred in the homogenate system. The effect of leaving out each component of the system is shown in Table V. The data clearly demonstrate the necessity of adding creatine, ATP, fluoride and the substrate. But, since the effect of adding cytochrome c and DPN to this system was not remarkable, we omitted these compounds. In the absence of the substrate some increase of in-

organic phosphate was observed as a result of the action of the tissue ATP-ase even though fluoride was present. So, in later experiments, we corrected the observed values for the released phosphate in the control experiments.

TABLE V

The Oxidation of α-Ketoglutarate and the Coupled Phosphorylation by the Homogenates of Normal Rat Hearts

(*a*) *The Influence of the Addition of NaF, Creatine, and ATP*

α-Keto-glutarate	ATP	Creatine	NaF	Rate of oxygen uptake (20 min. incubation) *μl.*	Inorganic phosphate balance (20 min. incubation) *μg.*
+	+	+	+	45.8	− 66
−	+	+	+	7.4	+ 90
+	−	+	+	23.3	− 20
+	+	−	+	45.8	− 12
+	+	+	−	130.8	− 24
−	+	+	−	22.8	+190

(*b*) *The Influence of the Addition of Cytochrome c, and DPN (NaF, Creatine, and ATP are added)*

α-Ketoglutarate	Cytochrome c, 0.1 % 0.1 ml.	DPN 0.01 *M* 0.1 ml.	Rate of oxygen uptake (20 min. incubation) *μl.*	Inorganic phosphate balance (20 min. incubation) *μg.*
+	+	+	70.0	−125
+	−	−	68.0	−105
−	+	+	10.0	+ 66
−	−	−	9.8	+ 66

Each Warburg vessel contained 0.5 ml. of 0.02 *M* Na-α-ketoglutarate, 0.5 ml. of 0.005 *M* K-ATP, 0.2 ml. of 0.1 *M* K-phosphate buffer, 0.2 ml. of 0.5 *M* KCl, 0.2 ml. of 0.1 *M* $MgCl_2$, 0.2 ml. of 0.25 *M* NaF, 30 mg. of creatine, 0.3 ml. of 16.7% homogenate, in a total volume of 3 ml., made up with water. Gas phase: air. The taps were closed after a 10-minute equlibration period, and the incubation took place during the next 10 minutes.

Thiamine in the Homogenates and the Esterification of Phosphate—Under the conditions stated above we observed in using the homogenized kidneys and hearts from thiamine-deficient rats what the relationship is between the phosphorylation of creatine and the oxidation of α-ketoglutarate when the thiamine content was the limiting factor in this system. The result is shown in Table VI.

TABLE VI

The Oxidation of α-Ketoglutarate and the Coupled Phosphorylation by the Homogenates of Rat Kidney and Heart (0.067 *M NaF*)

The figures in parentheses represent the values obtained in the case of using 0.167 *M* NaF.

	Rat No.	Heart			Kidney		
		O *microatom*	ΔP *microatom*	P:O	O *microatom*	ΔP *microatom*	P:O
Normal	12	3.6 (2.4)	2.8 (2.5)	0.7 (1.1)	3.9 (1.3)	4.1 (2.6)	1.1 (2.0)
	8	7.9 (4.5)	3.9 (3.6)	0.5 (0.8)	3.3 (1.5)	4.3 (3.5)	1.3 (2.3)
	21	1.6 (0.6)	2.4 (1.2)	1.5 (2.0)	1.2 (0.6)	1.5 (1.2)	1.3 (2.0)
	16	2.4 (2.0)	3.4 (2.6)	1.4 (1.3)			
	26				2.0 (0.6)	1.7 (1.1)	0.9 (1.8)
Pair-fed controls	11	1.7	1.9	1.3	2.0	1.8	0.9
	20	2.1	1.7	0.8	1.0	1.8	1.8
	25	1.6	1.4	0.9	3.9	4.2	1.1
	30	2.6 (1.9)	3.1 (4.0)	1.3 (2.1)	2.6	4.0	1.5 (2.5)
Thiamine-deficient	10	1.3	0.6	0.4	1.6	0.6	0.4
	18	0.8	0.5	0.6	2.0	1.0	0.5
	22	1.0	0.6	0.6	1.4	1.1	0.7
	23	1.4	0.6	0.4	1.2	0.8	0.6
	24	1.0	0.4	0.4	1.2	1.0	0.8
	28	0	0	0	0.9	0.6	0.6

The experimental conditions were the same as in Table V except the concentrations of NaF. O represents the oxygen uptake and ΔP represents the amount of inorganic phosphate which disappeared during the 20 minutes of the experiment.

When the final concentration of NaF was 0.167 *M*, known to be optimal in the normal rat homogenates, the oxygen uptake was re-

markably low in the thiamine-deficient and pair-fed rats, and the phosphate esterification in the thiamine-deficient tissues was so low that the accurate P:O ratio could scarcely be estimated in these tissues. Accordingly, we tried to determine the most adequate concentrations of NaF to be used in the experiments with normal, thiamine-deficient, and pair-fed rats. In the case of normal rats the highest P:O ratio was observed when the concentration of NaF was 0.167 *M*. When the concentration was 0.067 *M*, the P:O ratio was decreased. In the case of pair-fed rats and thiamine-deficient rats, however, the P:O ratio was not lower in 0.067 *M* NaF than in 0.167 *M* NaF. The oxygen uptake and the phosphate esterification could be measured accurately at this lower concentration of NaF. Consequently we used 0.067 *M* NaF for determining the P:O ratio in thiamine-deficient and pair-fed rats and both 0.067 *M* and 0.167 *M* NaF for determining that in normal rats. As shown in Table VI, in the thiamine-deficient hearts and kidneys the oxygen uptake was lower than that of the control rats tissues, but the phosphate uptake was lowered to such an extent the that P:O ratio was decreased markedly in them.

In the thiamine-deficient kidneys the addition of the mixture of catalytic amounts of diphosphothiamine and thiamine restored not only the oxygen uptake but the phosphorylation and also the values of the P:O ratio, as shown in Table VII. The effects of various concen-

TABLE VII

The Effect of Thiamine and Diphosphothiamine upon the Oxidation of α-Ketoglutarate and the Coupled Phosphorylation by the Homogenates of Thiamine-Deficient Rat Kidneys

(−): No addition, (+): Additions of 10 μg. of thiamine and 1 μg. of diphosphothiamine.

	O *microatom*	ΔP *microatom*	P:O	O *microatom*	ΔP *microatom*	P:O	O *microatom*	ΔP *microatom*	P:O
Rat No.	27			22			17		
(−)	0.45	0.4	0.9	0.9	0.58	0.6	1.38	1.06	0.7
(+)	0.55	1.1	2.1	1.3	1.0	0.8	1.45	1.5	1.1
Rat No.	18			24			28		
(−)	2.0	1.0	0.5	1.3	1.0	0.8	0.45	0.4	0.9
(+)	2.6	1.4	0.55	1.4	1.4	1.0	0.5	1.0	2.0

The experimental conditions were the same as in Table V (0.067 *M* NaF).

trations of diphosphothiamine and thiamine are shown in Table VIII. From these results it may be observed that diphosphothiamine plays an essential role in the oxidative phosphorylation by rat kidney and heart homogenates when α-ketoglutarate is added as the oxidizable substrate.

TABLE VIII

The Effect of Thiamine and Diphosphothiamine upon the Oxidation of α-Ketoglutarate and the Coupled Phosphorylation by the Homogenates of Thiamine-deficient Rat Tissues

(*a*) *Kidney*

Thiamine	Diphospho-thiamine	No. 28			No. 24		
		O	ΔP	P:O	O	ΔP	P:O
microgram	*microgram*	*microatom*	*microatom*		*microatom*	*microatom*	
0	0	0.45	0.4	0.9	1.3	1.0	0.8
10	1	0.5	1.0	2.0	1.4	1.4	1.0
10	5	0.6	1.3	2.1	1.6	1.6	1.0
10	10	0.9	1.3	1.5			
20	10				2.0	2.0	1.0
10	20	0.8	1.3	1.6			

(*b*) *Heart*

Thiamine	Diphospho-thiamine	No. 24			No. 27			No. 28		
		O	ΔP	P:O	O	ΔP	P:O	O	ΔP	P:O
microgram	*microgram*	*micro-atom*	*micro-atom*		*micro-atom*	*micro-atom*		*micro-atom*	*micro-atom*	
0	0	1.0	0.4	0.4	0.4	0.3	0.7	0	0	(0)
10	1	1.7	0.8	0.5						
10	3							0.5	0.2	0.4
10	5	2.0	1.4	0.7	2.0	1.2	0.6	0.3	0.7	2.3
10	10							0.5	0.9	1.8
10	20							0.7	0.9	1.3

The experimental conditions were the same as in Table V (0.067*M* NaF).

Under the above experimental conditions almost all the disappeared α-ketoglutarate was converted to succinic acid, when the concentration of NaF was 0.167 *M* for normal tissues and 0.067 *M* for thiamine-defici-

ent tissues and pair-fed controls. This fact was confirmed by comparing of the amount of oxygen consumed with that of α-ketoglutarate disappeared.

DISCUSSION

In most animal tissues α-ketoglutarate undergoes oxidative decarboxylation to produce succinic acid and carbon dioxide, when tissue succinic dehydrogenase is blocked by a proper concentration of malonate. Under this experimental condition diphosphothiamine seems to be necessary for the α-ketoglutarate oxidation system of animal tissue, because the smaller is the thiamine content of the tissue, the lower is the rate of the oxygen uptake of the tissue in the presence of α-ketoglutarate. Moreover, the addition of catalytic amounts of diphosphothiamine and thiamine restored somewhat the oxygen uptake in the avitaminous tissue homogenates. From the experimental results using the tissue slice technique, Barron and Lyman (*9*) stated that diphosphothiamine was necessary for the oxidation of α-ketoglutarate in animal tissues. However, Ochoa (*1*) could not succeed in separating diphosphothiamine from his α-ketoglutaric dehydrogenase preparation of cat heart muscle and reported that the addition of diphosphothiamine to the preparation produced only a small effect on the rate of the oxidation of α-ketoglutarate. Recently Stumpf, Zarudnaya, and Green (*2*) reported that α-ketoglutaric oxidase from pigeon breast muscle required diphosphothiamine and that this prosthetic group could be split off from the particles containing the enzyme. Our present findings make it more certain that the oxidation of α-ketoglutarate in animal tissues requires diphosphothiamine.

As for cytochrome c, Ochoa (*1*) stated that ferricytochrome c was rapidly reduced by α-ketoglutarate in the presence of his dehydrogenase. There is no information at present regarding whether flavoprotein and pyridine nucleotide are necessary for the oxidation of α-ketoglutarate to succinate. In the present experiment there were no observable effects from the addition of cytochrome c or DPN to our homogenate. Our preparation probably contained sufficient quantities of these two components as well as flavoprotein, since the animals were fed with nutritionally complete rations except that thiamine was lacking. The necessity of ATP for the oxidation of α-ketoglutarate was reported by Ochoa, and our findings coincide with his observations.

The coupling of phosphorylation with the oxidation of α-ketogluta-

rate was reported by Ochoa (*1*), Potter (*10*), Cross, Taggart, Covo and Green (*14*), and Hunter and Hixon (*6*). But there is no report on the relationship between phosphorylation and the thiamine content in animal tissues. Our results showed that the activity of the oxidative phosphorylation in the tissue homogenates of the kidneys and hearts of thiamine-deficient rats also was decreased when α-ketoglutarate was added as the oxidizable substrate. And it is a noticeable fact that the P:O ratio in the thiamine-deficient rats was remarkably lower than that in the normal or pair-fed control rats. The addition of catalytic amounts of both diphosphothiamine and thiamine restored not only the oxygen uptake but also the phosphate esterification, and the P:O ratio in the avitaminous rats. This fact suggests that diphosphothiamine is an essential component of the enzyme system for the oxidative phosphorylation in these tissue homogenates. From the physiological standpoint the tissue oxidation is of value only when it provides energy to be ultilized by the tissues. So it should be a very important fact from the standpoint of tissue metabolism that the tissue homogenates of thiamine-deficient rats cannot effectively produce high-energy phosphate bonds by the transformation of α-ketoglutarate to succinate through oxidation.

SUMMARY

1. It was demonstrated that the KCl isotonic homogenates of liver and kidney from thiamine-deficient rats shown in the presence of α-ketoglutarate and malonate, lower rate of oxygen consumption than the homogenates obtained from normal or pair-fed control rats.

2. In the thiamine-deficient liver homogenates there was some correlation between $Qo_2(N)_{\alpha\text{-ketoglutarate}}$ and thiamine content in the tissue.

3. The mixture of catalytic amounts of diphosphothiamine and thiamine restored the rate of the oxidation of α-ketoglutarate by the homogenates of thiamine-deficient rats to some extent.

4. The phosphate uptake in kidney and heart homogenates of thiamine-deficient rats coupled with the early stage of α-ketoglutarate oxidation was markedly low and the P:O ratio was also low as compared with those of normal or pair-fed control rats.

5. The mixture of catalytic amounts of diphosphothiamine and thiamine restored the oxidative phosphorylation and the P:O ratio to some extent.

6. These data support the view that diphosphothiamine plays an important role in the enzyme system of the tissue homogenates which brings about the oxidation of α-ketoglutarate to succinate and the coupled phosphorylation.

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STUDIES ON THE METHOD FOR DIFFERENTIAL MICRODETERMINATION OF BILE ACIDS IN BILE

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Cholic, desoxycholic and chenodesoxycholic acids are aboundunt, and physiologically as well as pathologically important in four human bile acids. These acids are present as conjugated bile acids in normal condition. However, as reported by Schoenheimer, Andrews and Herdina (*1*) and others, unconjugated bile acids are found in bile in some pathological cases.

The fact that the amount of free bile acids, particularly of dihydroxycholanic acid, plays an extremely important role in the formation of gall stone, has been frequently asserted by Miyake (*2*) and others.

Many methods for differential determination of bile acids have been reported (*2*, *3*, *4*, *5*, *6*). But these methods have some defects to determine bile acids in human bile. The author made an experiment in order to devise a new method for simple and rapid determination of the amounts of the conjugated and free human bile acids separately, using a small quantity of material. For separating the conjugated from free bile acid, the author has utilized the difference in solubility in ether between them, and for differential determination of trihydroxycholanic acid and dihydroxycholanic acid the author attempted to employ Kawaguchi's modification (*8*) of Abe's method (*7*). This method, however, was found to be inadequate in some respects after repeated examinations. It was, therefore, obliged to modify the method, more or less, to serve as a more suitable and accurate method for determination of bile acids in human bile. Separation of desoxycholic acid from chenodesoxycholic acid was so difficult that in this report only the total quantity of both unseparated will be given.

EXPERIMENTAL

I. *The Method for Determination of Total Bile Acids:*

Bile Acids Used.—The bile acids used for the examination were all

presented by Prof. T. Shimizu of the Medical Department, Okayama University.

Procedure for Color Development and Preparation of Extinction-Concentration Curve.

Reagents:

(a) Concentrated phosphoric acid. A colorless pure home product, ascertained not to contain NO_3 by a qualitiative examination with diphenylamine.

(b) Phosphoric acid I. A water dilution of the above concentrated phosphoric acid with a specific weight of 1.625 at 22°.

(c) Phosphoric acid II. Above pure phosphoric acid with a specific weight of 1.750 at 22°.

(d) Vanillin phosphoric acid solution I. Pure vanillin is dissolved in phosphoric acid I in a ratio of 1.0 mg. of vanillin to 1.5 ml. of phosphoric acid.

(e) Vanillin phosphoric acid solution II. Pure vanillin is dissolved in phosphoric acid II in a ratio of 1.0 mg. of the former to 1.5 ml. of the latter.

Serial amounts of alcoholic solutions of pure bile acids were taken into a number of conic flasks, and alcohol was evaporated to dryness under reduced pressure in a water bath at a temperature of about 50°. 1.5 ml. of vanillin phosphoric acid solution I were then dropped into one of the flasks. Shaking from time to time so as to bring bile acid into full contact with the reagent, the flask was kept in a water bath at a temperature of 50° for exactly 15 minutes. The presence of trihydroxycholanic acid is shown by red color reaction by this time, that of desoxycholic acid and its conjugated acid by a faint reddish violet when it was contained in the amount of 1.0 mg. at least, while the mixture was colorless under 0.8 mg. Chenodesoxycholic acid and its conjugated acid were quite colorless even when it contained to 1.0 mg. Next, after being cooled in running water for 1 minute 1.0 ml. of phosphoric acid I was added to it and fully mixed, extinction of the colored solution by the reaction was at once measured with Pulfrich's stufenphotometer using S_{53} filter. Then 1.5 ml. of vanillin phosphoric acid solution II were dropped into another flask and heated in the same manner as the former for 15 minutes in a water bath maintained at 70°. By this time trihydroxycholanic acid was colored red tinged with orange, desoxycholic acid a little dark reddish violet, and chenodesoxycholic acid beautiful reddish violett. Then it was cooled in running water for 1 minute,

TABLE I

Extinctions-coefficient of Pure Bile Acids

Amount of bile acid	Extinction-coefficient					
	Vanillin phosphoric acid solution I, 50°, 15 min.		Vanillin phosphoric acid solution II, 70°, 15 min.			
	Sodium cholate	Glycocholic acid	Sodium cholate	Glycocholic acid	Glycodesoxycholic acid	Glycohenodesoxycholic acid
mg.						
0.05	——	——	——	——	0.11	0.11
0.075	0.11	0.10	0.095	0.085	0.16	0.16
0.1	0.145	0.135	0.125	0.115	0.21	0.205
0.2	0.285	0.26	0.24	0.225	0.41	0.4
0.3	0.44	0.385	0.36	0.335	0.61	0.6
0.4	0.56	0.52	0.475	0.445	0.82	0.8
0.5	0.71	0.635	0.595	0.555	1.02	0.98
0.6	0.84	0.77	0.705	0.655	1.22	0.18
0.8	1.14	1.005	0.95	0.865	1.63	1.42
1.0	1.37	1.23	1.145	1.03	2.04	——

TABLE II

Extinction of a Mixed Solution of Glycocholic Acid and Glycodesoxycholic Acid Reacted with Vanillin Phosphoric Acid Solution I at 50° for 15 minutes

Glycocholic acid	Glycodesoxychoilic acid	Extinction of mixed solution	
		Calculated	Determined
mg.	*mg.*		
0.1	0.8	0.133	0.135
0.2	0.8	0.26	0.263
0.3	0.8	0.382	0.385
0.4	0.8	0.516	0.514
0.5	0.8	0.632	0.628

1.0 ml. of phosphoric acid II was added and the extinction was read as in the former case. The results of pure bile acids using the above stated procedure are summarized in Table I. It is clear that the Beer's law holds good. The results on the mixed solution of two kinds of bile acids are tabulated in Tables II and III.

TABLE III

Extinction of a Mixed Solution of Glycocholic Acid and Glycochenodesoxycholic Acid Reacted with Vanillin Phosphoric Acid Solution II at 70° for 15 minutes

Glycochenodesoxycholic acid	Glycocholic acid	Extinction of mixed solution	
		Calculated	Determined
mg.	*mg.*		
0.05	0.4	0.552	0.55
0.075	0.4	0.598	0.61
0.1	0.4	0.644	0.648
0.2	0.4	0.842	0.818
0.3	0.4	1.041	1.026

They show that the extinction of the mixture of two bile acids is nearly equal to the sum of the values of each separate acid, promising the possibility of differential determination by this method.

Between cholic acid and glycocholic acid the author could not recognize the difference of extinction described by Nagaki (*9*). The difference between the two given in Table I originates only in their difference in molecular weight. This is probably the case with dihydroxycholanic acid.

Selection of Optimum Conditions—A number of experiments were performed to determine the optimum conditions for application of this color reaction as a method of quantitative analysis. These experiments may be summarized as follows: (a) Quantity of vanillin and phosphoric acid—In Kawaguchi's method, the quantity of vanillin was too great compared with that of bile acid, and drying up the alcoholic solution of vanillin could hardly be performed uniformely every time, so that each determination does not give the uniform result. Vanillin was therefore reduced from 8 mg. in Kawaguchi's method to 1 mg. and dissolved in phosphoric acid. The concentration of phosphoric acid was the same as in the original method, but the quantity taken

was 2.5 ml. to meet the capacity of the cuvette of the stufenphotometer. Consequently, the intensity of the color developed by the same quantity of bile acid became remarkably lower than in Kawaguchi's method, but as shown in Table IV, the author became aware that the extinction

TABLE IV

Comparison of Extinction between One Stage- and Two Stage-Usages of Phosphoric Acid

Type and amount of bile acid	Amount of vanillin	Temperature and time of reaction	Extinction	
			One stage usage of phosphoric acid	Two stage usage of phosphoric acid
mg.	*mg.*	*minutes*		
Glycocholic acid 0.6	2.0	70°, 10	0.70	0.96
Glycocholic acid 1.0	2.0	50°, 10	0.88	1.25
Cholic acid 0.4	2.0	50°, 10	0.44	0.62
Cholic acid 0.1	2.0	50°, 10	0.16	0.18

was greater when the color reaction was performed using 1.5 ml. of phosphoric acid first and 1.0 ml. of phosphoric acid was added, a definite period later than when 2.5 ml. of phosphoric acid was used at a time. The author therefore preferred the present method, using a reagent in which 1.5 ml. of phosphoric acid contained 1.0 mg. of vanillin; (b) Extinction curve—In Fig. 1 is given the extinction curve of the colored solution developed by the reaction in different periods of time between the same quantity of cholic acid and vanillin phosphoric acid solution I in a water bath at a temperature of 50°. It represents the color nuance changing according to the different periods of reaction. Likewise, Figs. 2, 3, and 4 give the extinction curves in the case of the reaction of vanillin phos-

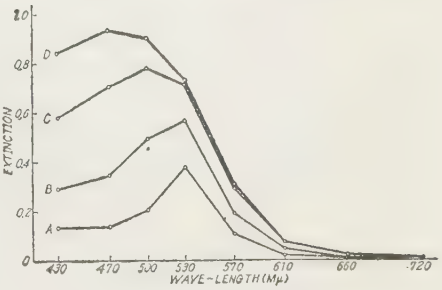

FIG. 1. Extinction curve for the pigment produced from cholic acid in the modified vanillin-phosphoric acid reaction when vanillin phosphoric acid solution I was used at 50°. (The reaction time: Curve A, 7 min.; Curve B, 10 min.; Curve C, 15 min.; Curve D, 20 min.)

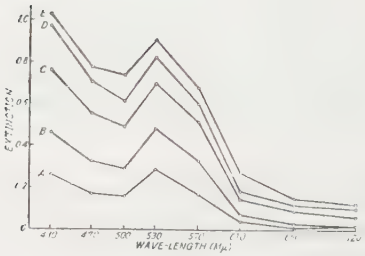

FIG. 2. Extinction curve for the pigment produced from glycodesoxycholic acid when vanillin phosphoric acid solution II was used at 70°. (The reaction time: Curve A, 5 min.; Curve B, 8 min.; Curve C, 15 min.; Curve D, 20 min.; Curve E, 30 min.)

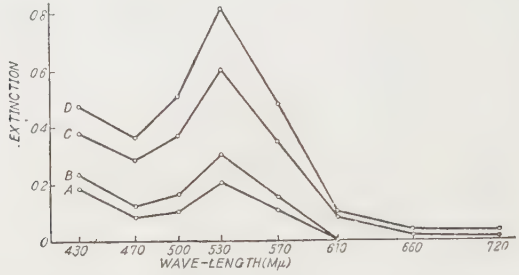

FIG. 3. Extinction curve for the pigment produced from glycochenodesoxycholic acid when vanillin phosphoric acid solution was used at 70°. (The reaction time: Curve A, 5 min.; Curve B, 8 min.; Curve C. 15 min.; Curve D, 20 min.)

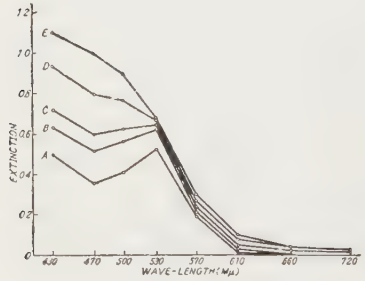

FIG. 4. Extinction curve for the pigment produced from cholic acid when vanillin phosphoric acid solution II was used at 70°. (The reaction time: Curve A, 5 min.; Curve B,; 8 min.; Curve C, 10 min.; Curve D, 15 min.; Curve E, 20 min.)

phoric acid solution II to glycodesoxycholic acid, glycochenodesoxycholic acid and, cholic acid, respectively. The curve of glycocholic acid was just the same as that of cholic acid; (c) Optimum temperature, reaction time and filter—The temperature indicated in the original method was adopted. The reaction time was decided as 15 minutes in every case in view of the above extinction curve, and the fact that

between chenodesoxycholic acid and desoxycholic acid there was marked difference in extinction when kept at 70° for 8 minutes, but near similarity when kept at 70° for 15 minutes. The filter used as the fittest was S_{53} in view of the extinction curve; (d) Change in extinction after color development—Fig. 5 represents a change in extinction occurring at room temperature after the above-stated reaction. When vanillin phosphoric acid solution II was put to act on dihydroxycholanic acid and trihydroxycholanic acid, the color developed was stable, but there was a rather quick change in the color when vanillin phosphoric acid solution I was reacted with trihydroxycholanic acid, so that the extinction should be measured quickly soon after the reaction; (e) Specificity of

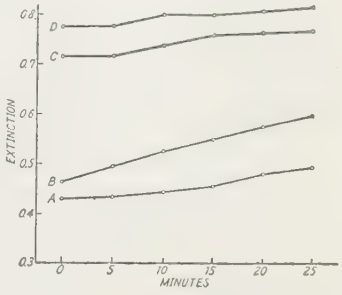

FIG. 5. Changes in extinction of the colored solutions during periods of standing at room temperature after completion of the color-developing reaction. (Curve A for cholic acid, Curve C for glycodesoxycholic acid, Curve D for glycochenodesoxycholic acid, each reacted with vanillin phosphoric acid solution II at 70° for 15 min. Curve B was that of cholic acid reacted with vanillin phosphoric acid solution I at 50° for 15 min.)

color reaction—Concerning the specificity of this color reaction, there are detailed descriptions by Abe, Chabrol *et al.* (*10*) and a few others. The author reexamined the discordant points in these reports, and the result obtained was about the same as Abe's. Oleic acid was, however, colored faintly yellowish brown, but it was contained in bile in such a small quantity that it did not practically interfere with the actual determination of bile acid. Protein needed to be removed thoroughly as it turned red in this reaction.

II. *Separation of the Free Bile Acids from the Conjugated.*

A definite quantity of pure bile salts dissolved in .4 ml. of Walpole's acetate buffer (pH 4.8) was taken into a separating funnel with capacity of about 150 ml., and then extracted twice each time with 10 ml. of redistilled ether saturated with water. After the buffer layer was separated completely from the ether phase, the buffer was collected together and the quantity of bile acids remaining in this solution was measured by the above mentioned procedure. The ratio of the remaining bile acids in the buffer, as measured by this method using pure

bile acid was below 2.5% for each free bile acid, 81±2 % for glycocholic acid and 80±2 % for dihydroxycholanic acid conjugated with glycine as shown in Table V. The result of the examination of the gallbladder

TABLE V

Experiment of Ether-Extraction

Remaining ratio of bile acids in buffer after ether extraction. W is abbreviation of Walpole's acetate mixture, and K is that of Kolthoff's borax-citrate mixture, both at pH 4.8

Kind of bile salt	Amount of bile salt used	Kind of buffer	Remaining amount of bile salt in buffer	
	mg.		*mg.*	*per cent*
Cholate	10	W	0.24	2.40
	20	W	0.49	2.45
	10	K	0.25	2.50
	20	K	0.41	2.05
Glycocholate	10	W	8.18	81.8
	20	W	16.26	81.3
	10	K	8.06	80.6
	20	K	16.15	80.75
Desoxycholate	10	W	0.15	1.50
Glycodesoxycholate	10	W	7.96	79.6
	20	W	16.04	80.2
Glycochenodesoxycholate	10	W	8.2	82.0
Cholate + Glycocholate	10 10	W	8.2	——

bile of dogs by this method is shown in Table VI, indicating that taurine-conjugated bile acids remain in the buffer in the ratio of 80.2±2.5 % for trihydroxycholanic acid, and 81.5±2.5 % for the mixed solutions of trihydroxycholanic acid and dihydroxycholanic acid. It means that this method is sufficiently applicable in quantitative separation of the

TABLE VI

Remaining Ratio of Bile Acid Conjugated with Taurine in Buffer after Ether-Extraction

In every cases, 1 ml. of 1:200 diluted alcoholic solution of canine gall bladder bile was used for color reaction

Dog	Extinction-coefficient			
	Vanillin phosphoric acid solution I. 50°, 15 min.		Vanillin phosphoric acid solution II. 70°, 15 min.	
	Before ether-extraction	After ether-extraction	Before ether-extraction	After ether-extraction
I	0.50	0.40	0.98	0.81
II	0.53	0.42 0.415	1.10	0.90
III	0.52	0.42 0.43	1.11	0.89

free bile acids from the conjugated, the error being below 5% when the bile acids remaining in the buffer after extraction with ether are regarded as conjugated bile acids altogether.

As represented in Table V, this method always brings the same results irrespective of the kind of buffer provided its pH is unchanged.

III. *Author's Method for Differential Analysis of Bile Acid in Bile:*

For decolorization and deproteinization, to an aliquot quantity of bile in a flask are added several times of 96% alcohol, make alkaline the mixture with a few drops of ammonia, to which 10–20 mg. of norit is added, and shaken thoroughly. The flask is placed in a water bath kept at 80–90° for 2 or 3 minutes, then cooled at room temperature and filtered. The residue is washed several times with small quantity of alcohol. If the filtrate is clear after being left overnight, it is made up to a definite volume with alcohol before it is used. If the filtrate is turbid and contains some sediments, it is boiled again for 2–3 minutes, refiltered, and then made up to a definite volume with alcohol. The deproteinization should be made completely, because protein gives red-color by this reaction of vanillin and phosphoric acid. No serious error affecting the result comes about even though decolorization is not absolutely complete. An aliquot quantity from this solution, which has been made to a definite volume, is taken into 2 conic flasks, and

alcohol is driven off by the aforesaid method; one of them is then subjected to react to vanillin phosphoric acid solution I at 50° for 15 minutes, and the other to vanillin phosphoric acid solution II at 70° for 15 minutes; and the extinction is measured with Pulfrich's stufenphotometer. In this procedure, the former shows the extinction of trihydroxycholanic acid, and the latter the sum of the extinction of trihydroxycholanic acid and that of dihydroxycholanic acid. An approximate estimate of quantity of bile acid is thus obtained. Next, an aliquot portion is taken from the remaining filtrate, and after complete evaporation of alcohol and water from it under reduced pressure, the dry residue is dissolved in 4 ml. of Walpole's acetate buffer (pH 4.8). This mixture is taken into a separating funnel for separation of the free bile acid from the conjugated by the above mentioned method. After being left intact till the buffer layer becomes transparent enough, the buffer is separated from ether and the bile acid remaining in the buffer is determined by the above mentioned procedure. The obtained result gives the quantity of conjugated bile acids, and the difference between this quantity and the quantity determined previously is that of free bile acids. The amount of bile acid is calculated from the following fomula:
The amount of conjugated trihydroxycholanic acid

$$=0.1\times\frac{E_2\mathrm{Tri}}{0.1277}\times\frac{100}{80}(\mathrm{mg.})$$

The amount of conjugated dihydroxycholanic acid

$$=0.1\times\frac{E_2\mathrm{Mix}-(E_2\mathrm{Tri}\times 0.89)}{0.2}\times\frac{100}{80}(\mathrm{mg.})$$

Amount of cholic acid $=1.0\times\dfrac{E_1\mathrm{Tri}-\left(E_2\mathrm{Tri}\times\frac{100}{80}\right)}{0.1417}(\mathrm{mg.})$

Amount of free dihydroxycholanic acid

$$=0.1\times\frac{E_1\mathrm{Mix}-\left(E_2\mathrm{Mix}\times\frac{100}{80.}\right)}{0.216}(\mathrm{mg.})$$

E_1Tri is extinctions-coefficient of mixture when vanillin phosphoric acid solution I is used at the time of determination of total bile acids; E_1Mix is extinctions-coefficient of mixture when vanillin phosphoric acid solution II is used at the time of determination of total bile acids; E_2Tri is extinctions-coefficient of mixture when vanillin phosphoric acid solution I is used at the time of determination of conjugated bile acids; E_2Mix is extinctions-coefficient of mixture when vanillin phosphoric acid solution II is used at the time of determination of conjugated bile acids.

IV. *Results of the Determination of Bile Acids in Human Bile and Recovery Test.*

Results of the analysis of bile acid in human gallbladder bile obtained by laparotomy, and of recovery test in which pure bile acids were added to that bile, are shown in Table VII. As shown in Table VII, the percentage of recovered pure bile acid is 99.3±3 %, and the quantity of conjugated bile acid calculated from the recovery in the buffer after the extraction with ether, is 101.2±2 % for trihydroxycholanic acid, and 100.6±2.5 % for dihydroxycholanic acid, against the value determined by the procedure without the ether-extraction.

SUMMARY

Reexamination of Kawaguchi's modification of Abe's method, utilizing the reaction of bile acid with vanillin and phosphoric acid disclosed some defects of the method as a means of quantitative determination of human bile acids in bile, and the author undertook all sorts of experiments in order to establish more accurate method, which might be applicable to human bile. The author further studied a simple method of quantitative separation of free bile acids from the conjugated by extraction with ether. Then the author devised the following method of analyzing bile acid by the combination of both.

1. Desoxycholic acid and chenodesoxy cholic acid are measured together as two substances can not be determined separately when they are mixed.

2. Hence, the quantity of trihydroxycholanic acids and that of total dihydroxycholanic acids in human bile are determined separately, one as the free acid and the other as the conjugated acid.

3. The range of possible determination is 0.075–1.0 mg. for trihydroxycholanic acid, 0.05–1.0 mg. for desoxycholic acid, and 0.05–0.6 mg. for chenodesoxycholic acid.

4. The recovery of bile acid added to human bile is 99.3—3 %.

5. The quantity of the conjugated bile acid determined by the ether—extraction method corresponds to 101.2—2% for trihydroxycholanic acid, and 100.6—2.5 % for dihydroxycholanic acid, of the value obtained by the method without ether extraction.

6. The quantity of bile required is very small, the procedure is simple, and will be finished in a short time.

Table VII

Result of the Determination of Bile Acid in Human Gallbladder Bile and Recovery Test

In the column of conjugated bile acid, the upper and lower values represent the results of the procedure, without and with ether-extraction, respectively.

Cases	Amount of bile	Type and amount of bile acid added	Conjugated bile acid determined *mg./ml.*		Free bile acid determined *mg./mg.*		Recovery
			Tri-hydroxy-cholanic	Di-hydr-oxycho-lanic	Tri-hydroxy-cholanic	Di-hydr-oxycho-lanic	
	ml.	*mg.*					*per cent*
K.S. (Cholecystitis chron. sine concremento)	1.0	0	13.1 13.5	15.5 15.3	0	0	——
,,	,,	Glycocholate 10.0	22.9 23.3	15.6 15.3	0	0	98.0
,,	,,	Cholate 10.0	—— 13.1	15.6 16.2	9.7	0	97.0
D.K. (Carcinoma ventriculi)	1.0	0	43.0 43.8	48.0 49.0	0	0	——
,,	,,	Cholate 20.0	—— 44.0	47.7 48.8	20.4	0	102.0
S.K. (Cholecystitis chron. sine concremento)	1.0	0	42.0 43.0	24.9 35.0	0	0	——
,,	,,	Cholate 20.0	—— 42.5	24.9 24.2	20.5	0	102.5
S.M. (Cholecystitis chron. sine concremento et pancreatitis chron.)	1.0	0	12.5 12.8	—— 20.8	0	3.0	——
,,	,,	Glycodesoxycholate 20.0	12.7 12.5	—— 40.3	0	3.2	97.5
,,	,,	Desoxycholate 10.0	—— 12.5	—— 20.9	0	12.6	96.0
,,	,,	Glycocholate 20.0	33.0 32.8	—— 20.4	0	3.0	100.0
E.H. (Cholecystitis chron. sine concremento)	1.0	0	15.0 15.5	14.3 14.3	0	0	——
,,	,,	Glycochenodesoxycholate 10.0	15.0 14.7	24.2 24.0	0	0	99.0

In conclusion, the author would like to express his sincere thanks to Prof. H. Miyake for his kind suggestion and criticism throughout this reserch, and Prof. T. Shimizu of Okayama University, Prof. K. Kodama of Tokyo University for their kind criticism. This reserch was subsidized by the Scientific Research Fund of the Ministry of Education.

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KINETIC STUDIES ON THE ACTION OF GLUCOSE DEHYDROGENASE. I

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It has been shown in previous papers (*1*, *2*, *3*) that the glucose dehydrogenase of *Aspergillus oryzae* oxidizes D-glucose in the presence of suitable hydrogen-acceptors, and that the reaction can proceed without the participation of co-enzyme and phosphate. The reaction is practically irreversible and may be represented by

D-Glucose + hydrogen-acceptor → D-gluconate + reduced acceptor

Though the molecular oxygen cannot directly be used as the hydrogen-acceptor, it does function as the ultimate hydrogen-acceptor when a suitable redox-dye such as thionine is present as an intermediary hydorogen-carrier. This paper describes the kinetic studies of an enzyme reaction with particular reference to the dependency of reaction rate upon the concentrations of enzyme, substrate and hydrogen-acceptor.

METHODS

The enzyme material employed was the same as that used in previous works (*2*, *3*). The oxidation rate of glucose was measured either manometrically by the amount of O_2-uptake (in the presence of thionine) or by the time of decolorization of redox-dyes using Thunberg's technique. Special technique was adopted when *p*-quinone was used as the H-acceptor.

The reaction mixtures used in the manometric measurements were of the following composition: in the main vessel, 1.0 ml. of buffered enzyme solution (10 mg. of enzyme preparation per 1.0 ml. of 0.1 *mole/lit.* phosphate buffer) and 1.0 ml. of thionine solution; in the side bulb: 0.5 ml. of D-glucose solution. In the experiments with Thunberg tubes, 0.5 ml. of buffered enzyme solution (0.1 *mole/lit.* phosphate buffer) was placed in the main tube, and 1.0 ml. of D-glucose solution and 0.5 ml. of $2 \times 10^{-3.0}$ *mole/lit.* solution of redox-indicator (thionine, *o*-cresol-indo-2,6-

dichlorophenol or 2,6-dichlorophenol-indophenol) in the side bulb. After repeated evacuation and refilling with N_2- or H_2-gas, the tubes were evacuated and submerged in a constant temperature bath. When thermal equilibrium was attained, the reaction was initiated by introducing the content of the side bulb into the main tube. The reduction rate was calculated from the time required, not for total decolorization, but for decrease of the oxidized form of the dyestuff to a definite low concentration: *e.g.*, $5 \times 10^{-5.0}$ *mole/lit.*

The experiments using *p*-quinone as hydrogen-acceptor were carried out as follows: Three ml. of D-glucose solution and 1.0 ml. of 0.01 *mole/lit.* *p*-quinone solution were placed in a 50 ml. flask which was provided with a rubber stopper having inlet and outlet tubes. The outer ends of the tubes had short lengths of rubber tubing and screw-clamps attached. In the flask a small test tube (3.0 ml. in volume) containing 1.0 ml. of buffered enzyme solution (1 mg. enzyme preparation in 1.0 ml. of phosphate buffer) was placed. After aerating the flask with N_2- or H_2-gas for five minutes, the inlet and outlet tubes were closed and the reaction was initiated by introducing the content of the small tube into the main space of the flask. After a definite interval the reaction was stopped by the addition of 5.0 ml. of 2 *N* sulfuric acid, then 5.0 ml. of 5% KI solution was added and the amount of iodine liberated was titrated with 0.01 *N* sodium thiosulfate solution.

All the experiments were carried out at pH 7.2 and at 30°.

RESULTS

The Reaction Rate as a Function of the Concentration of Enzyme—The effect of enzyme concentration on the rate of oxidation was investigated manometrically using 0.4 *mole/lit.* glucose as the substrate and 0.02 *mole/lit.* thionine as the hydrogen-carrier. As will be shown in later experiments, these concentrations of substrate and H-acceptor were both saturating for the activity of the enzyme. It was found that the O_2-uptake during 15 minutes, as shown in Fig. 1, increased quite lineally with the increase of the enzyme concentration. When the concentration of enzyme (ε) is given in terms of [*g./lit.*], the amount of glucose oxidized (or dyestuff reduced) [*mole/lit.*] per minute per unit concentration of the enzyme was

$$0.54 \times 10^{-4} \left[\frac{mole}{lit.}\right] \Big/ \left[\frac{g.}{lit.}\right] [min.]$$

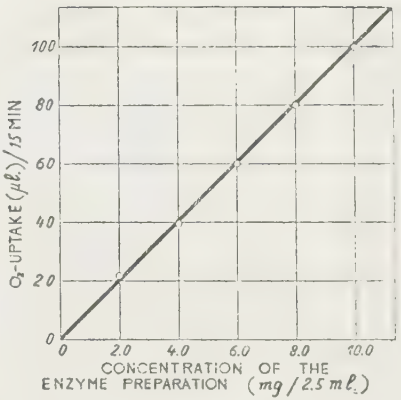

FIG. 1. O_2-uptake in the presence of enzyme in various concentrations.

Composition of the reaction mixture: 1.0 ml. of buffered enzyme solution; 1.0 ml. of 0.05 *mole/lit.* thionine; 0.5 ml. of 2 *mole/lit.* D-glucose. Temp., 30°.; pH 7.2.

The Reaction Rate as a Function of the Concentration of Hydrogen-Acceptor—Using various concentrations of thionine and 2,6-dichlorophenol-indophenol, the reduction rate was compared by Thunberg's technique. The results obtained are summarized in Table I in which the rate is given, as was proposed above, in terms of $\left[\frac{mole}{lit.}\right]/\left[\frac{g.}{lit.}\right]$ [*min.*]. From

TABLE I

Effect of the Initial Concentration of Dyestuffs on the Rate of Reaction

Buffered enzyme solution (2 mg./ml.), 0.5 ml.; thionine or 2.6-dichlorophenol-indophenol (2×10^{-3} *mole/lit.*), 1.0 ml., 0.8 ml., 0.6 ml., 0.4 ml., or 0.2 ml.; D-glucose solution (1 *mole/lit.*) 0.5 ml. Total volume of reaction mixture was made up to 2.0 ml. with distilled water. Temp.: 30°; pH: 7.2

Rate of reaction $\left[\frac{mole}{lit.}\right]/\left[\frac{g.}{lit.}\right][min.]$ — Initial concentration of dyestuff [*mole/lit.*] / Redox-indicator	10^{-3}	0.8×10^{-3}	0.6×10^{-3}	0.4×10^{-3}	0.2×10^{-3}
2,6-Dichlorophenolindophenol	1.24×10^{-4}	1.24×10^{-4}	1.24×10^{-4}	1.24×10^{-4}	1.24×10^{-4}
Thionine	0.51×10^{-4}	0.50×10^{-4}	0.51×10^{-4}	0.50×10^{-4}	0.50×10^{-4}

this table it will be seen that for both redox-dyes studied the difference in the initial concentration in the range from 0.2×10^{-3} to 10^{-3} *mole/lit.* had no effect upon the rate of decolorization of the dyestuffs (up to the concentration of $5 \times 10^{-5.0}$ *mole/lit.*). Experiment using *p*-quinone as the hydrogen-acceptor, which we shall not reproduce here to save space, also showed that the reduction rate was independent of the concentration of acceptor in the range from 0.01 to $10^{-4.0}$ *mole/lit.*

Experiment was also made to measure the rate of O_2-uptake in the presence of thionine in different concentrations: 0.02, 0.01 and 0.007 *mole/lit.* (concentration of glucose added: 0.4 *mole/lit.*). The rate of O_2-uptake remained constant within the range of concentration studied. The oxidation velocity computed from these data was $0.50 \times 10^{-4.0}$ $\left[\frac{mole}{lit.}\right] / \left[\frac{g.}{lit.}\right]$ [*min.*], a value which coincides well with other data obtained with thionine as hydrogen-acceptor. This coincidence indicates that in this experiment the non-enzymatic reoxidation of leuco-dyestuff has not been a rate determining step.

At any rate, it may be concluded that at least up to the concentration studied (redox-dyestuffs 5×10^{-5} *mole/lit.*; *p*-quinone 10^{-4} *mole/lit.*) the reaction may be regarded as being of the zero order in respect to the concentration of the hydrogen-acceptor.

The Reaction Rate as a Function of the Concentration of Substrate—The effect of substrate concentration upon the O_2-uptake in the presence of saturating concentration of thionine (0.02 *mole/lit.*) was investigated manometrically. The initial concentration of glucose applied was $1.0 \times 10^{-2.0}$, $1.0^{-2.0}$ and $0.5 \times 10^{-2.0}$ *mole/lit.* As is apparent from Fig. 2, the rate of oxidation is dependent on the glucose concentration, and the oxidation ceased at the stage corresponding to the total disappearance of the glucose. From the curves it may be seen that the time (τ) required for half consumption of the substrate is practically the same (68, 60 and 60 minutes, respectively), indicating that in the range of concentration studied the reaction was of the first order in respect to the substrate concentration. The O_2-uptake was measured in the presence of 0.01 *mole/lit.* glucose and 0.02 *mole/lit.* thionine using various concentrations of the enzyme. In Fig. 3 are presented the time courses of the decrease of glucose concentration in logarithms, which had been calculated from the amount of oxygen consumed. That the reactions were of the first order in respect to the concentration of glucose may also be concluded from the linear relationship obtained. It may further be noticed that the tangent

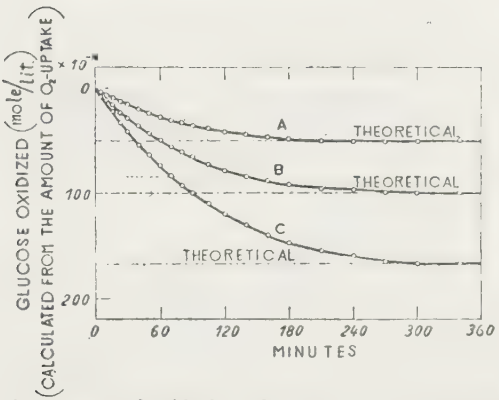

Fig. 2. Time course of oxidation of glucose given in three different initial concentrations.

The time (τ) of half consumption of glucose was in all cases about 60 minutes.

Composition of the mixture: 1.0 ml. of buffered enzyme (10 mg. per 1.0 ml.); 1.0 ml. of 0.05 *mole/lit.* thionine; 0.5 ml. of D-glucose. The concentration of D-glucose: (A) 0.005 *mole/lit.*, (B) 0.010 *mole/lit.*, and (C) 0.0167 *mole/lit.* Temp.; 30°,; pH, 7.2.

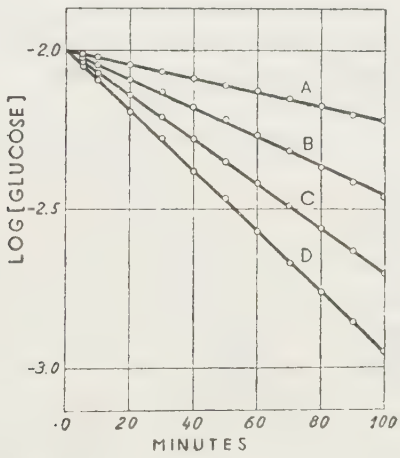

Fig. 3. The log[S]-t-curves.

Ordinate: the logarithm of the concentration of D-glucose at a given time, which was calculated from O_2-uptake.

Abscissa: reaction time.

The composition of the mixture: 1.0 ml. buffered enzyme solution, 1.0 ml. 0.05 *mole/lit.* thionine and 0.5 ml. 0.05 *mole/lit.* D-glucose.

Amount of the enzyme in the mixture: (A) 5 mg., (B) 10 mg., (C) 15 mg., and (D) 20 mg.

of the curves in Fig. 3 is proportional to the concentration of the enzyme applied.

Experiments to be described below showed that in the presence of a definite concentration of the enzyme the initial rate of the oxidation was related to the concentration of glucose according to the well-known Michaelis equation (*4*):

$$v/V_m = \frac{[S]}{M+[S]} \qquad (1)$$

where v is the initial rate, V_m the maximum rate to be observed at a sufficiently high concentration of the substrate, and **M** the Michaelis constant. When thionine was used as the hydrogen acceptor, the maximum rate V_m was observed at the glucose concentration of 0.4 *mole/lit.*

Effect of the Hydrogen-Acceptors upon the Maximum Rate V_m and the Michaelis Constant—A series of experiments was carried out to determine the effect of hydrogen-acceptors upon the functional relationship between the rate of oxidation and the substrate concentration [S]. Hydrogen-acceptors used were thionine, *o*-cresol-indo-2,6-dichlorophenol, 2,6-dichlorophenol-indophenol and quinone, which were all given in saturating concentrations. Most of the experiments were run using anaerobic methods; only with thionine both aerobic and anaerobic measurements were carried out, which, however, were found to give essentially the same results.

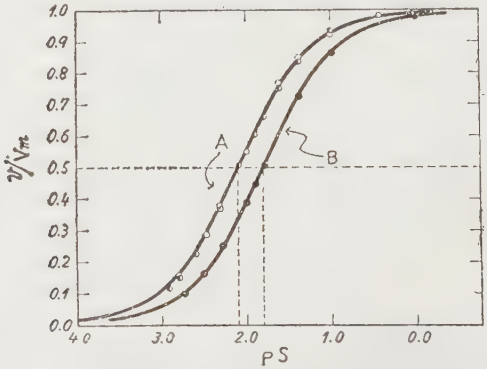

FIG. 4. Effect of redox-dyestuffs upon the v/V_m-pS-relationship. Substrate: D-glucose. ○: observed value calculated from 0_2-uptake in initial 5 min., in presence of thionine, (A); ◐: observed value calculated from the time of 90% decolorization of thionine, (A); ●: observed value calculated from the time of 90% decolorization of 2,6-dicholorophenol-indophenol, (B). Temp., 30°.; pH 7.2.

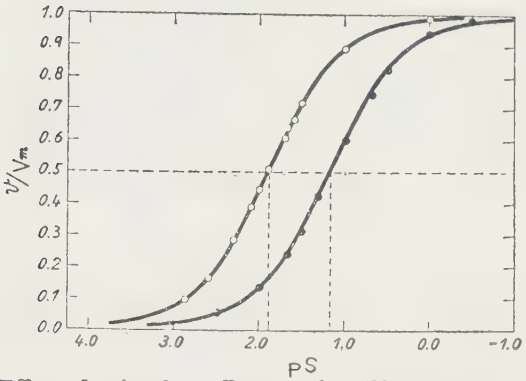

FIG. 5. Effect of redox-dyestuffs upon the v/V_m-pS-relationship. Substrate: D-glucose. o: *o*-Cresol-indo-2,6-dichlorophenol as H-acceptor. •: *p*-Quinone as H-acceptor. Temp., 30°.; pH, 7.2.

The results obtained are summarized in Figs. 4 and 5, in which the ratio v/V_m is plotted against the logarithm of the substrate concentration ($pS = -\log_{10}[S]$). In all cases sigmoid curves were obtained which were satisfactorily in accordance with the Michaelis equation. From these curves the Michaelis constants were determined as the value of [S] corresponding to $v/V_m = 0.5$. The values of **M** and V_m/ε (ε being the concentration of enzyme, see below) obtained are listed in Table II, from which it may be seen that both these values vary according to the nature of the hydrogen-acceptors.

TABLE II

The Values of V_m/ε *and the Michaelis Constant for* D-*glucose*
(Temperature 30°.; pH: 7.2)

Redox-indicator	Thionine	*o*-Cresol-indo-2,6-dichloro-phenol	2,6-Dichloro-phenol-indo-phenol	*p*-Quinone
$\frac{V_m/\varepsilon}{\left[\frac{mole}{lit.}\right]} \Big/ \left[\frac{g}{lit.}\right] [min.]$	0.5×10^{-4}	0.95×10^{-4}	1.24×10^{-4}	7.0×10^{-4}
M [*mole/lit.*]	0.0085	0.0126	0.0150	0.0670

As previously reported (*3*), the enzyme used in our expriments can also act upon 6-phospho-D-glucose. Using this substance as a sub-

strate, the rate-substrate concentration-relationship was studied by anaerobic method in the presence of saturating concentration of thionine or 2,6-dichlorophenol-indophenol. As was the case with glucose, the v/V_m-pS-relationship obtained was in accordance with the Michaelis equation, which is represented by the sigmoid curve in Fig. 6. In Table

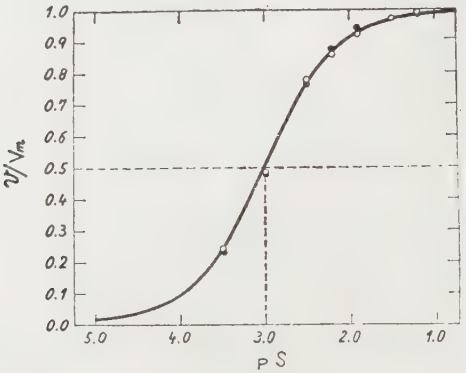

Fig. 6. v/V_m-pS-curve for 6-phospho-D-glucose.
o: Thionine as H-acceptor.
•: 2,6-Dichlorophenol-indophenol as H-acceptor.

III are given the values of V_m/ε and **M** obtained, which are apparently different from those found for glucose.

Table III

The Values of V_m/ε *and the Michaelis Constant for 6-Phospho-D-Glucose.* (Temp., 30°., pH: 7.2)

Redox-indicator	Thionine	2,6-Dichlorophenol-indophenol
$\left[\frac{mole}{lit.}\right]/\left[\frac{g}{lit.}\right][min.]$	0.54×10^{-5}	0.65×10^{-5}
M [*mole/lit.*]	0.001	0.001

DISCUSSION

To account for various facts observed in our experiments—in which the hydrogen-acceptors have always been applied in saturating con-

centration — we propose the following scheme as representing the mechanism of the enzyme reaction*.

$$E + F \underset{k_1'}{\overset{k_1}{\rightleftarrows}} EF \qquad (2)$$

$$EF + S \underset{k_2'}{\overset{k_2}{\rightleftarrows}} EFS \qquad (3)$$

$$EFS \xrightarrow{k_3} E + P + H_2F \qquad (4)$$

where E is the free enzyme, F the hydrogen-acceptor, S the substrate, P the product, and EF and EFS, the complexes formed of the molecules indicated. The value k assigned to each formula means the rate constant of each reaction.

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* In general terms, the reaction would be described as follows:

$$E + F \underset{k_1'}{\overset{k_1}{\rightleftarrows}} EF$$

$$E + S \underset{k_2'}{\overset{k_2}{\rightleftarrows}} ES$$

$$EF + S \underset{k_2'}{\overset{k_2}{\rightleftarrows}} EFS$$

$$ES + F \underset{k_1'}{\overset{k_1}{\rightleftarrows}} EFS$$

$$EFS \xrightarrow{k_3} E + P + H_2F$$

It can be shown, however, that this scheme leads to the same results as the one given above, in so far as we restrict our consideration to the phenomena occurring in the presence of saturating concentration of F. As a possible scheme we may also picture the following sequence of reactions:

$$E + S \rightleftarrows ES$$
$$ES + F \rightleftarrows EFS$$
$$EFS \rightarrow E + P + H_2F$$

or

$$E_0 + S \rightleftarrows E_0S$$
$$E_0S \rightarrow E_r + P$$
$$E_r + F \rightleftarrows E_rF$$
$$E_rF \rightleftarrows E_0 + H_2F$$

where E_0, and E_r represent the oxidized and the reduced forms, respectively, of the enzyme molecule. It can easily be shown that these schema are irreconcilable with the experimental data.

Stationary state kinetics based on the above scheme lead to the following equation:

$$v=\frac{k_3\varepsilon}{\frac{k_1'(k_2'+k_3)+k_2k_3[S]}{k_1k_2[S][F]}+\frac{k_2'+k_3}{k_2[S]}+1} \qquad (5)$$

where ε is the total concentration of enzyme, *i.e.*,

$$\varepsilon=[E]+[EF]+[EFS]$$

In our experiment, the rate was found to be of the zero order in respect to the concentration of F. Under the condition used in our experiment, the first term in the denominator of Eq. (5) must have been negligibly small compared to the others. If it is so, the rate is given by

$$v=\frac{k_3\varepsilon}{\frac{k_2'+k_3}{k_2[S]}+1} \qquad (6)$$

which is in accordance with Eq. (1) already obtained experimentally. The Michaelis constant has, therefore, the following implication.

$$\mathbf{M}=\frac{k_2'+k_3}{k_2}$$

If $[S]\gg\mathbf{M}$,

$$V_m=k_3\varepsilon \qquad (7)$$

If $[S]\gg\mathbf{M}$,

$$v=\frac{k_2k_3\varepsilon[S]}{k_2'+k_3} \qquad (8)$$

The fact established in our experiment that with different hydrogen-acceptors different values of V_m/ε were obtained indicates that the rate constant k_3 is dependent on the nature of hydrogen-acceptor. The question now arises whether or not the values k_2' and k_2, or k_2'/k_2 (dissociation constant of reaction (3)) would also be dependent on the nature of hydrogen-acceptor. Eq. (8) may be rewritten in the form:

$$\frac{[S]\varepsilon}{v}=\frac{\mathbf{M}\cdot\varepsilon}{V_m}=\frac{k_2'}{k_2}\times\frac{\varepsilon}{V_m}+\frac{1}{k_2}$$

If k_2 and k_2' are independent of the nature of hydrogen-acceptor, the value $[S]\varepsilon/v$ observed under the condition of $[S]\ll\mathbf{M}$ must be a linear function of ε/V_m. Using the data illustrated in Figs. 5 and 6, and Table II the values of $[S]\varepsilon/v$ obtained with sufficiently low concentration of substrate (glucose) are plotted against ε/V_m for different kinds of hydrogen-acceptors. As may be seen from the figure, a linear relationship

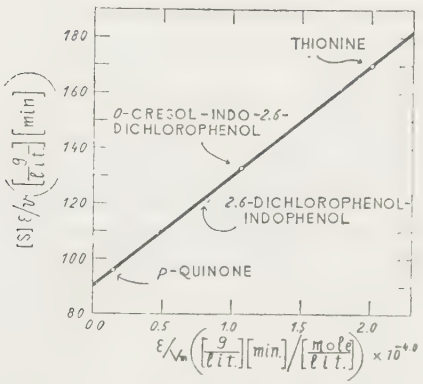

FIG. 7. The relation between the values $[S]\varepsilon/v$ and ε/V_m.

is approximately fulfilled, indicating that the values k_2 and k_2' are practically independent of the nature of the hydrogen-acceptor. From the tangent of the line obtained, the value k_2'/k_2 was estimated to be 0.004 *mole/lit.*

A further check on the correctness of the above conclusion is provided by the following consideration. As was shown already, different hydrogen-acceptors gave different values of **M** and k_3. Assuming k_2' and k_2 to be independent of hydrogen-acceptors, the Michaelis constant shown by different hydrogen-acceptors may be written as follows:

$$\mathbf{M}^{(I)}=\frac{k_2'+k_3^{(I)}}{k_2} \quad \text{and} \quad \mathbf{M}^{(II)}=\frac{k_2'+k_3^{(II)}}{k_2}$$

where the superscripts I and II refer to different hydrogen-acceptors. Then we have

$$\frac{\mathbf{M}^{(I)}k_2-k'_2}{\mathbf{M}^{(II)}k_2-k_2'}=\frac{k_3^{(I)}}{k_3^{(II)}}=\frac{\boldsymbol{V}_m^{(I)}}{\boldsymbol{V}_m^{(II)}}$$

or

$$K=\frac{k'_2}{k_2}=\frac{\boldsymbol{V}_m^{(I)}\mathbf{M}^{(II)}-\boldsymbol{V}_m^{(II)}\mathbf{M}^{(I)}}{\boldsymbol{V}_m^{(I)}-\boldsymbol{V}_m^{(II)}} \qquad (9)$$

By applying the values of $\boldsymbol{V}_m/\boldsymbol{\varepsilon}$ and **M** obtained with different hydrogen-acceptors, the value of k_2'/k_2 was calculated according to Eq.(9). The values obtained for all combinations of the hydrogen-acceptors studied were

$$k_2'/k_2=0.004 \ [mole/lit.] \text{ at } 30°.$$

This fact indicates that the dissociation constant ($K=k_2'/k_2$) of reaction (3) is independent of the nature of the hydrogen-acceptor combined with the enzyme.

The relative value of k_2' may be estimated from the values of V_m/ε, **M** and K according to the following equation:

$$k_2' = \frac{K \cdot \frac{V_m}{\varepsilon}}{\mathbf{M} - K}$$

From this and the value of K given already, we can also determine the relative value of k_2. In Table IV are summarized the relative values of k_2' and k_2 thus obtained. It may be seen that not only the dissociation constant, but also the rate constants of reaction (3) are independent of the nature of the hydrogen-acceptors combined with the enzyme molecule.

TABLE IV

Redox-indicators used	Relative value of k_2 $[lit./g]/[min.]$	Relative value of k_2' $\left[\frac{mole}{lit.}\right]/\left[\frac{g}{lit.}\right][min.]$
Thionine	$1.1\times10^{-2.0}$	$4.4_4\times10^{-5.0}$
o-Cresol-indo-2,6-dichlorophenol	$1.1\times10^{-2.0}$	$4.4_2\times10^{-5.0}$
2,6-Dichlorophenol-indophenol	$1.1\times10^{-2.0}$	$4.5_1\times10^{-5.0}$
p-Quinone	$1.1\times10^{-2.0}$	$4.4_4\times10^{-5.0}$

SUMMARY

1. In continuation of the studies reported earlier, the action of the glucose dehydrogenase isolated from *Aspergillus oryzae* was investigated in detail from a kinetic point of view.

2. It was shown that the following reaction scheme fits in well with all experimental data obtained under the conditions studied, *i.e.* in the presence of saturating concentration of a hydrogen-acceptor.

$$\begin{aligned} E + F &\rightleftarrows EF \\ EF + S &\rightleftarrows EFS \\ EFS &\rightarrow E + P + H_2F \end{aligned}$$

where E denotes the free enzyme molecule, F the hydrogen-acceptor, EF a complex in which the hydrogen-acceptor is bound reversibly to

E, EFS another complex in which substrate is bound reversibly to EF, P the reaction product, and H_2F the reduced form of hydrogen-acceptor.

3. Based on the data obtained with different hydrogen-acceptors the dissociation constant (K) of the reaction between enzyme molecule and substrate was calculated by the following equation:

$$K=\frac{\boldsymbol{V}_m^{(I)}\mathbf{M}^{(II)}-\boldsymbol{V}_m^{(II)}\mathbf{M}^{(I)}}{\boldsymbol{V}_m^{(I)}-\boldsymbol{V}_m^{(II)}}$$

where $\boldsymbol{V}_m$ represents the maximum velocity of overall reaction to be observed at saturating concentration of substrate, **M** the Michaelis constant, and the superscripts I and II refer to different hydrogen-acceptors. Applying this equation to the data obtained with different hydrogen-acceptors, the dissociation constant of D-glucose-enzyme-complex was estimated to be 0.004 *mole/lit.* (at 30°).

4. It was found that the hydrogen-acceptor combined with the enzyme molecule has no effect not only upon the dissociation constant, but also upon the rate constants of the reaction in which the substrate S combines with the complex EF.

In conclusion the writer expresses his cordial thanks to Prof. H. Tamiya for his constant encouragement and suggestion during the course of this research. Thanks are also due to Prof. K. Okunuki of the University of Osaka for a supply of the Robinson-ester used in this study. The cost of this research has been defrayed by the Grant in Aid for Fundamental Scientific Research of the Ministry of Education, which is gratefully acknowledged here.

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STUDIES ON THE CONJUGATED LIPIDS*
III. ON THE CONFIGURATION OF SPHINGOMYELIN

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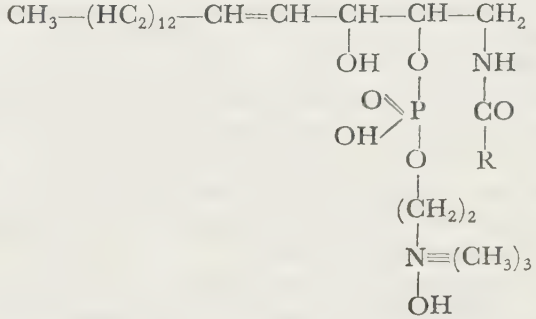
(Received for publication, September 10, 1951)

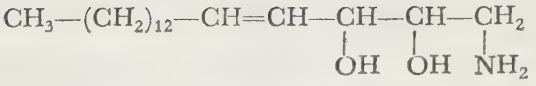
Sphingomyelin is characterized as a diaminophosphatide which is found only in the animal body. This substance was discovered for the first time in human brain by Thudichum (*1*). It was found to contain phosphoric acid, fatty acids and two bases, choline and sphingosine, as the constituents of its molecule by Thudichum, Rosenheim and Tebb (*1*), and especially by Levene (*2*).

For the structure of sphingomyelin Levene (1916) (*2*) presented the following constitutional formula (**I**) judging from its components. This assumption was supported by the configuration of sphingosine (**II**), which was reported later by Klenk and Diebold (*3*). Until recent time, the structure of sphingomyelin, however, has not been fully investigated.

(**I**)



(**II**)



* Two articles on this subject were previously published in *The Journal of Biochemistry* by Nakayama of this laboratory.

In studying the configuration of sphingomyelin, there was no question concerning choline phosphoric acid or phosphorylcholine and fatty acids themselves, but the constitution of sphingosine and the position at which fatty acid and phosphorylcholine combines with sphingosine remained unsettled.

Regarding the configuration of sphingosine, Ohno (1944) (*4*) and Carter (*5*) had independently tried the oxidation of sphingosine and its derivative with periodic acid and lead tetraacetate, respectively, and reached the same conclusion that the two hydroxyl radicals and one amino radical are arranged in contradiction to the view taken by Klenk and Diebold (**II**), as follows:

(**III**) $CH_3—(CH_2)_{12}—CH=CH—\underset{OH}{CH}—\underset{NH_2}{CH}—\underset{OH}{CH_2}$

Note. *i*) Cis-trans isomerism owing to the double bond in $C_{4\triangle5}$ is trans-type (Ohno (1944) (*4*)).

ii) Stereoisomers owing to the two asymmetric carbon atoms at C_2 and C_3 are not yet studied.

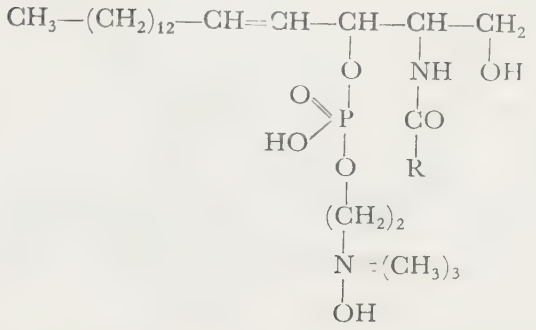
As regards the linkage between fatty acid and sphingosine, Levene (1916) (*2*) had obtained lignocerylsphingosine as an intermediary product of the imperfect hydrolysis of sphingomyelin. He found that this compound gave no nitrogen gas on treatment with nitrous acid and did not form any salt with mineral acids. It was concluded consequently that fatty acid forms the peptidic linkage with sphingosine at the C_2 position of the latter.

The position where phosphorylcholine is esterified with sphingosine remained unknown. Accordingly, the present author attempted to investigate this point as to sphingomyelin molecule whereby he referred a great deal to the method by which Nakayama (*6*) had elucidated the position of the glycosidic linkage between sphingosine and galactose in the cerebron molecule.

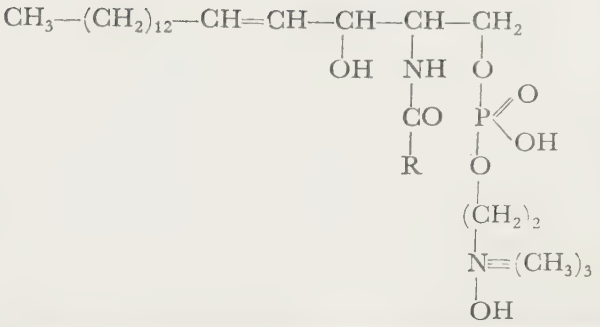
Since the configuration of sphingosine was decided as described above (**III**), the constitutional formula of sphingomyelin is to be either (**IV**), or (**V**). In this paper, the author wishes to determine which of the two formulae is correct.

If sphingomyelin be methylated, monomethylsphingomyelin would be obtained, since the sphingomyelin molecule contains only one hydroxyl group. By the hydrolysis of this compound, 1-O-methylsphingosine is produced from (**IV**), and 3-O-methylsphingosine from

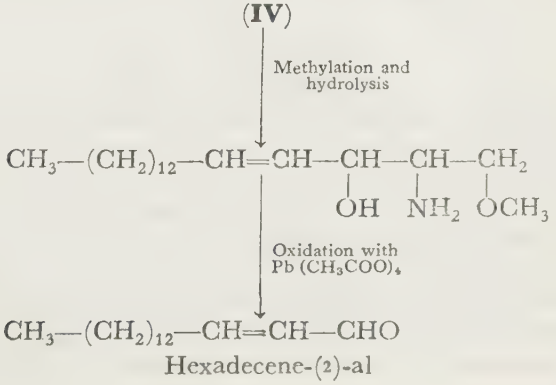
(**IV**)

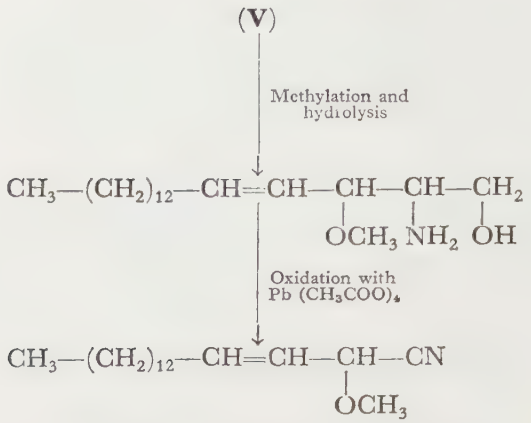


(**V**)



(**V**) as below. Applying the oxidation method to them with lead tetra-acetate after Criegee (*7*) according to Ohno (1948) (*4*) and Nakayama (*6*), one could obtain a C_2 unsaturated palmitic aldehyde from the former, and a C_3 unsaturated nitrile of seventeen carbon chains including one of the O-methyl group at C_2 position from the latter:





2-Methoxy-heptadecene-(3)-nitrile

Therefore, the esterified position between phosphorylcholine and sphingosine can be decided by observing the product obtained by hydrolysis and oxidation. Accordingly, the author hydrolyzed first the methyl derivative of the purified sphingomyelin and then studied especially methoxysphingosine, one of the hydrolysis products, in detail.

Note. Sphingomyelin obtained from pig brain contains nervonic acid, stearic acid, and lignoceric acid together as fatty acids. In the present report, the theoretical values were calculated as lignocerylderivatives.

EXPERIMENTAL

Isolation of Sphingmyelin from Pig Brain—This work was carried out mainly after the method of Levene (1914) (2) except the purification procedure. 2 kg. of the desiccated pig brain tissue were finely minced and extracted with an equal volume of boiling alcohol several times by using a Soxhlet apparatus. Each extraction was continued for about one hour. Extracted solution was gathered and left over night in a refrigerator. The precipitate formed on cooling was separated and then exhausitively extracted with ether and acetone. The residue was dissolved under stirring in 4 times volume of pyridine which had been warmed to 80°, and allowed to cool over night in a refrigerator. The precipitate formed on standing was separated and dissolved in 4 times volume of hot glacial acetic acid and allowed to cool at 0°. After the formed precipitate was separated off, the mother liquor was con-

centrated under diminished pressure and transferred into a large quantity of acetone. The precipitate thereby appeared was separated from the mother liquor and dried *in vacuo*. Thus, 34 g. of the crude sphingomyelin were obtained as a weak yellowish powder which was further purified by the following procedure:

The substance was dissolved in a large quantity of warm ligroin-alcohol (9:1) and left over night in an ice chest. The resulting precipitate was separated off, and the mother liquor was concentrated under diminished pressure and transferred into an equal volume of acetone to precipitate sphingomyelin which was separated and dried in a desiccator. The white powder thus obtained was further recrystallized repeatedly by dissolving in boiling pyridine-chloroform mixture and subsequent cooling. The ratio of pyridine to chloroform was changed so that the amount of pyridine was gradually increased against chloroform. That is, the powder was first dissolved in twice volume as much as pyridine-chloroform mixture (1:1) and recrystallized by cooling. The same treatment was repeated in 5 volumes of pyridine-chloroform mixture (4:1), and in 10 volumes of this mixture (9:1). Finally about 8.5 g. of the purified sphingomyelin were obtained. This substance was an amorphous snow white crystal, insoluble in acetone and ether, soluble in pyridine and alcohol to some extent, very easily soluble in chloroform-methanol mixture. Bial's orcin reaction for this substance was negative shwoing that cerebroside was not contaminated (m.p., 195°.)

Analyses of sphingomyelin: $C_{47}H_{97}N_2PO_7$

N:	found	3.76%
	calcd.	3.36%
(Substance: 28.800 mg.; *N*/50 NaOH: 3.83 ml.)		
P:	found	3.92%
	calcd.	3.72%
(Substance: 15.375 mg.; P: 0.603 mg.)		

Methylation of Sphingomyelin—Methylation was performed by making use of the method of Purdie and Irvine (*8*). 4 g. of the above purified sphingomyeline were dissolved in 10 ml. of chloroform-methanol, to which 15 ml. of pure methyl iodide and 5 g. of dry silver oxide were added gradually at intervals. Upon each addition the mixture was heated mildly in order to promote the reaction. At last the mixture was heated on a water-bath for thirty minutes under a reflux. After the mixture was left to cool, the precipitate was filtered off and the filtrate was evaporated to dryness. This treatment was repeated once more, and then

the residue was extracted with methanol. After removing methanol by evaporation, the residue was recrystallized in pyridine-chloroform mixture (1:1). Thus, 2.7 g. of methylsphingomyelin were obtained as amorphous snow white powder, which was insoluble in acetone, slightly soluble in ether and pyridine, and soluble in alcohol, methanol and chloroform, respectively.

Analysis of methylsphingomyelin $C_{48}H_{99}N_2PO_7$

OCH_3: found 3.61%
calcd. 3.66%
(Substance: 10.855 mg.; $N/100$ $Na_2S_2O_3$: 7.58 ml.)

Hydrolysis of Monomethylsphingomyelin—One and half g. of monomethylsphingomyelin were dissolved in 55 ml. of 10 per cent solution of methanolic sulfuric acid, hydrolyzed by boiling for eight hours on a water-bath under reflux, and left over night at 0°. Large amounts of methylesters of fatty acids precipitated on cooling were filtered off by suction and washed with a small amount of ice cold methanol. The filtrate was poured into about twice as much water, and heated on a water-bath to evaporate methanol. After cooling, the water solution was shaken several times in a separatory funnel with petroleum ether to remove a trace of fatty acid, then neutralized with dilute sodium hydroxide solution and left over night at 0°. The resulting viscous precipitate of sphingosine fraction, was separated by centrifuging at 2500 r.p.m. for one hour at low temperature.

Sphingosine fraction thus obtained was washed with a small amount of ice-cold water and dissolved immediately in 1 N alcoholic sodium hydroxide solution. Alcohol being evaporated under diminished pressure, the residue was suspended in a small amount of water, then added to ether and shaken in a separatory funnel. The ether layer was separated, dried with anhydrous sodium sulfate and evaporated under diminished pressure. The residue was dissolved in a small amount of absolute alcohol, follwed by the addition of absolute alcoholic sulfuric acid solution, the quantities of which had been calculated previously, drop by drop until the solution showed a faint acid reaction against litmus paper. This neutralized solution was cooled over night at 0°, and the resulting crystal of O-methylsphingosine sulfate was filtered and washed with a small amount of ice-cold absolute alcohol. The procedure by which O-methylsphingosine was precipitated after passing through the free base, was repeated several times. About 0.3 g. of this substance thus purified was obtained as snow white crystals.

Analysis of O-methylsphingosine sulfate: $(C_{19}H_{39}NO_2)_2 \cdot H_2SO_4$

OCH_3: found 7.75%

calcd. 8.56%

(Substance: 4.652 mg.; $N/100$ $Na_2S_2O_3$: 6.98 ml.)

Oxydation of O-Methylsphingosin with Lead Tetraacetate –A quarter g. of O-methylsphingosine sulfate above described was led to the free base again, desiccated sufficiently and dissolved in 10 ml. of anhydrous benzene. To this solution were added 0.4 g. of lead tetraacetate gradually at intervals. Then the solution was warmed at 50° for three hours under the reflux. After cooling, a small amount of ethylene glycol was added to decompose excess lead tetraacetate. Then the benzene was washed repeatedly with water in a separatory funnel to get rid of lead salt. The benzene layer was transferred to another flask and dried with anhydrous sodium sulfate. The residue, which was obtained after evaporating benzene under diminished pressure, did not show positive reaction for Schiff's reagents (fuchsin-sulfurous acid solution). The product thus obtained was assumed to be a nitrile. This porduct was therefore treated with 5 ml. of alcohol containing 0.4 g. of potassium hydroxide and boiled under the reflux on a water-bath for hydrolysis. When the boiling was continued for about three hours, the evolution of ammonia gas was noticed at the top of condenser. This boiling was further continued for ten hours after the reaction against Nessler's reagent had been shown negative. The reaction mixture was left to cool, added to alcoholic sulfuric acid solution—the amount of this solution being calculated before use—drop by drop, till the solution showed slight acid reaction against litmus paper. To this solution were added 40 ml. of water and it was placed on a water-bath to be freed from alcohol by evaporation. To the solution in which fatty acid was suspended was added ether in a separatory funnel. After being sufficiently shaken, the ether layer was separated, dried with anhydrous sodium sulfate and evaporated under diminished pressure. The oily residue was dissolved in methanolic ammonia solution. After excess methanol and ammonia had been evaporated, the ammonium salt of fatty acid was redissolved in methanol. To this solution was added methanolic silver nitrate solution drop by drop. Then white and cloudy precipitate formed, was separated and dried to yield about 40 mg. of silver salt of the fatty acid.

Analyses of silver salt of methoxyheptadecenic acid $C_{18}H_{33}O_3Ag$

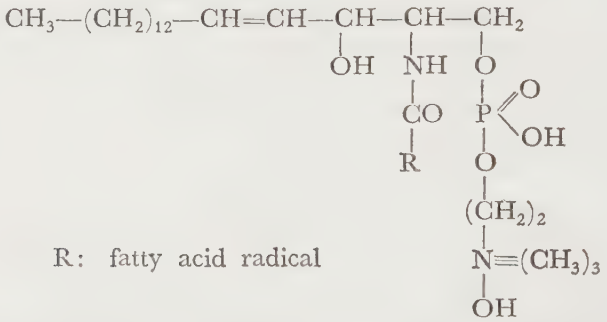
OCH_3: found 6.96%

calcd. 7.65%

(Substance: 4.545 mg.; $N/100$ $Na_2S_2O_3$: 6.12 ml.)
Ag: found 26.54%
calcd. 26.66%
(Substance: 5.935 mg.; Ag: 1.575 mg.)

SUMMARY

The author isolated the pure sphingomyelin from pig brain, then methylated it to monomethylsphingomyelin by the method of Purdie. The sphingosine fraction, one of the hydrolysis products of this methyl derivative, was treated with lead tetraacetate after Criegee, and was studied further in detail. The resulting substance was a silver salt of methoxyheptadecenic acid, which is to have been derived from 2-methoxyheptadecene-(3)-nitrile. It was proved therefore that the esterification of phosphorylcholine to sphingosine was effected at the C_1 position of sphingosine. Thus the author claims that the constitutional formula of sphingomyelin is to be expressed as follows:



Note. Stereoisomers due to the asymmetric carbon atoms at C_2 and C_3 must be studied further, as in the case of sphingosine.

The author wishes to express his thanks to Prof. Morio Yasuda for his advice and encouragement throughout the course of this work.

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The Journal of Biochemistry, Vol. 39, No. 1.

STUDIES ON THE CONJUGATED LIPIDS IV. ON THE ENZYMATIC HYDROLYSIS OF SPHINGOMYELINE

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(Received for publication, September 10, 1951)

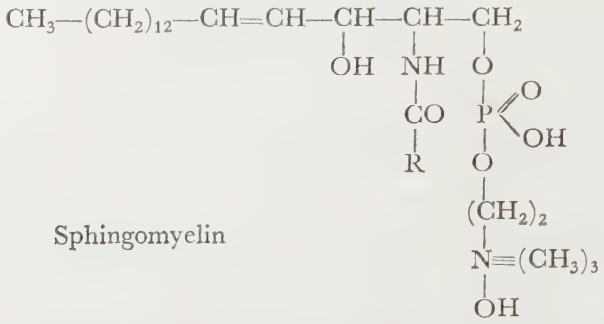
In order to comprehend the biological significance of the conjugated lipids it is essential to study the problem from enzymological angle. Some enzymological studies have been carried out regarding glycerolphosphatides, mainly lecithins, but the results of these studies only showed that glycerolphosphatides could be hydrolyzed not merely by chemical reagents, but also by using certain biological catalysts. The author believes, however, that the enzymological studies on glycerolphosphatide should be carried on hereafter, with acetalphosphatide, inositolphosphatide, phosphatidic acids and so forth as well, in order to elucidate their metabolic mechanisms, since these substances seem to be closely related with one another in animal body, judging from their chemical constitutions or their biological properties. The same may be said concerning the group of sphinoglipids; that is, the enzymological investigation on them should be attempted in consideration of the close relationship between the phosphosphingosides and the glycosphingosides.

Thanhauser and Reichel (*1*) tried an enzymatic hydrolysis of the cerebrosides and reported that cerebrosidase was active in the presence of proper activators to liberate glactose and that activators which stimulated cerebrosidase activity depressed the activity of polydiaminophosphatase. This study was, so far as the author's knowledge, the only one that has been performed from such a point of view as above mentioned.

The author has therefore attempted an enzymatic decomposition of sphingomyelin to get insight into the correlation between sphingomyeline and cerebroside, which has been known as the most common phosphosphingoside, and glycosphingoisde, respectively.

Sphingomyelin is distributed widely in the animal body, especially

rich in brain, and is always found together with cerebrosides as well as with other glycosphingosides, the constitutional formula after the present author (*2*) is shown below



Sphingomyelin

R: fatty acid radical; *i.e.*, lingoceryl, nervonyl, stearyl, and palmityl are identifiable.

From the above structure of sphingomyelin, it is assumed that the following three enzymes at least may participate in the enzymatic decomposition of sphingomyelin: (a) Cholinesterase liberating choline from the molecule of sphingomyelin; (b) phosphatase liberating inorganic phosphoric acid; (c) peptidase decomposing the peptidic linkage of fatty acid to sphingosine. Sphingomyelinase, if exists, would display its activity against sphingomyelin having the action of either one or two or all of these three enzymes.

Rossi (*3*) once tried the enzymatic hydrolysis of polydiaminophosphatide—polymer of sphingomyelin—, and reported that inorganic phosphoric acid was liberated almost completely from polydiaminophosphatide under the conditions devised successfully by him. It may be conceivable that the phenomenon observed in his study was the result of uaited actions of cholinesterase and phosphatase. It is not elucidated accordingly, which enzyme of these two acts more rapidly than the other; that is to say, the problem remains unsettled whether inorganic phosphoric acid is liberated from the sphingomyelin molecule after choline has been freed fom it, or phosphorylcholine is first liberated and further split into phosphoric acid and choline. Furthermore, the action of the peptidase has not been studied yet. The author has tried to contribute to these problems.

For this purpose, the experimental result of Rossi was first con-

firmed and then the process of enzymatic hydrolysis was investigated. In the first study, the suitable condition for enzymatic action was established, which accorded with the conditions reported by Rossi except that the composition of the reaction-mixture was modified to some extent. Details of this point are therefore not described in this paper. In the second study, the following substances were determined at definite time intervals under the optimal conditions; liberation of inorganic P, acid-soluble P, choline and amino-N.

Sphingomyelinase was prepared from pig spleen and the activity was determined by estimating each hydrolysis product.

EXPERIMENTAL

Preparation of Enzyme Solution—Enzyme solution was prepared virtually according to the method adopted by Klein to prepare the nucleosidase (*4*). Spleen of young pig, taken out immediately after slaughter and still warm, was cut in slices as quickly as possible and then frozen in a mixture of ether and dry ice to liberate the adhering enzyme in cells. These frozen pieces of the organ were dried completely in a vacuum desiccator. Sufficient desiccation was attained in a week at most. After these desiccated pieces had been pulverized and the combined fibers of the organs removed by passing through a sieve, the fine powder placed in a firmly stoppered bottle, was preserved in a refrigerator. This powder maintained its original enzymatic activity for one year at least.

Three g. of this powder were suspended in 100 ml. of physiological sodium chloride solution and shaken vigorously for seven or eight hours under occasional cooling and then centrifuged at low temperature. The supernatnant colloidal solution was transferred into a collodium-bag and dialyzed against running water for three days and against distilled water for a day, successively. In this process inorganic P in the bag was completely removed. The liquid in the bag was centrifuged once more, and then stored with a small quantity of toluen in an ice chest as the enzyme solution, which was used for hydrolysis of sphingomyelin. This enzyme solution maintained its original enzymatic activity for a month at least.

Preparation of Substrate Solution—Crude sphingomyelin obtained from pig brain after Levene (*5*) was purified as described in the previous report until Bial's orcin reaction became negative. 1.000 g. of this substance was dissolved in 100 ml. of physiological sodium chloride solution and shaken vigorously for two or three hours. The shingomyelin was emulsified homgeneously, but this colloidal solution often

formed a cloudy bulk during its storage, which could be avoided by the addition of 50 mg. of sodium desoxycholate. Salts of bile acids are effective, as is well known, in stabilizing the solution of lipids and in preventing the substrate from salting-out by magnesium salt, which is used as an activator of sphingomyelinase. The solution thus obtained was almost clear and stable. It was stored in an ice chest under a layer of petroleum ether.

Determination of Enzymatic Activity—Enzymatic hydrolysis was carried out in the reaction-mixture containing the following components in the proportion indicated: Substrate solution (1% solution of sphingomyelin) 2 parts; buffer solution (HCl-veronal buffer at pH 7.9) 3 parts; enzyme solution prepared as above) 5 parts. A small quantity of toluen was occasionally added to this reaction-mixture during hydrolysis in order to prevent undesirable decomposition. After being stoppered, the mixture was held in an incubator and left to react to almost complete hydrolysis. At definite intervals, an aliquot of this mixture was taken out and added to the same volume of 20% trichloroacetic acid solution to interrupt the enzymatic reaction. After ten minutes, the protein was perfectly precipitated, and filtered off. Using this filtrate, each product of hydrolysis was determined. Inorganic P and acid-soluble P were estimated by Bodansky's colorimetric method (*6*), choline by Beatie's colorimetric method (*7*), and amino-N by van Slyke's method.

The control experiment was performed with substrate and enzyme solution respectively in parallel with this main experiment. In the control test using substrate only, no decomposition was observed, whereas in that of enzyme only, hydrolysis products such as inorganic P, acid-soluble P and amino-N were liberated to some extent, although in most cases in negligibly small amount due to autolysis. Accordingly, these autolysis values of enzyme were subtracted from the main test. Thus, the enzyme activity was indicated in terms of the quantity of each hydrolysis product in 10 ml. of the reaction-mixture.

Hydrolysis of Sphingomyeline—The products liberated during hydrolysis were estimated every twenty-four hours under the following conditions. Each figure represents mg. of hydrolysis product in 10 ml. of the reaction-mixture.

Sphingomyelin was almost completely hydrolyzed under these conditions in six or seven days, that is, as shown in the following table, approximately 95% of inorganic P and acid-soluble P, and about 92% of

Conditions of Hydrolysis:—pH, 7.9; Temp., 37°; Mg-salt as activator: 0.02 *M* of the mixture

Time \ Hydrolysis product	Acid-soluble P	Inorganic P	Choline	Choline / P	Amino-N
1 *Days*	0.495 *mg.*	0.297 *mg.*	— *mg.*	—	0 *mg.*
2	0.585	0.485	1.842	3.8	0
3	0.655	0.570	2.280	4.0	—
4	0.732	0.675	2.635	3.9	−0.011
5	0.750	0.712	2.775	3.9	—
6	0.758	0.750	2.926	3.9	0.011
7	0.758	0.743	2.482	4.0	0.011
Value of 100% hydrolysis*	0.782	0.782	3.228	4.1	0.326

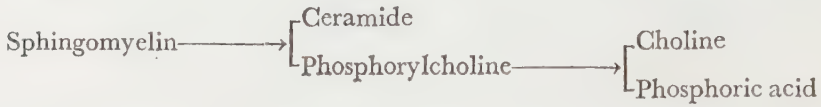
* Theoretical value was calculated as lignoceryl-sphingomyelin $C_{47}H_{97}N_2PO_7$ (832.84).

choline were liberated at the end of hydrolysis. Lower liberation of choline seems to be based on technical errors in the experimental method. Acid-soluble P showed always higher value than inorganic P at the beginning until the middle of the course of hydrolysis. Both values, however, became to coinside fairly well when hydrolysis proceeded further. This fact shows that phosphorylcholine was contained in this reaction-mixture together with inorganic phosphoric acid, since acid-soluble P consists of inorganic P and organic P, meaning phosphoryl-choline-P in the case of this experiment. The ratio of choline to inorganic P was almost constant and agreed with the calculated value, indicating that choline and inorganic P were formed at the same time in the process of hydrolysis. These results demonstrate therefore that phosphoryl-choline was first liberated, which was split into choline and inorganic phosphoric acid in the next stage.

On the other hand, amino-N was liberated only from the enzyme solution, and not from the substrate sphingomyelin at all. This fact shows that amino-group was not freed from the sphingomyelin molecule, that is, shingosine combined tightly with fatty acid as ceramide which was not affected by this enzymatic activity.

DISCUSSION

From the experimental results shown above, it seemes possible that the enzymatic hydrolysis of sphingomyeline proceeds along the following course:

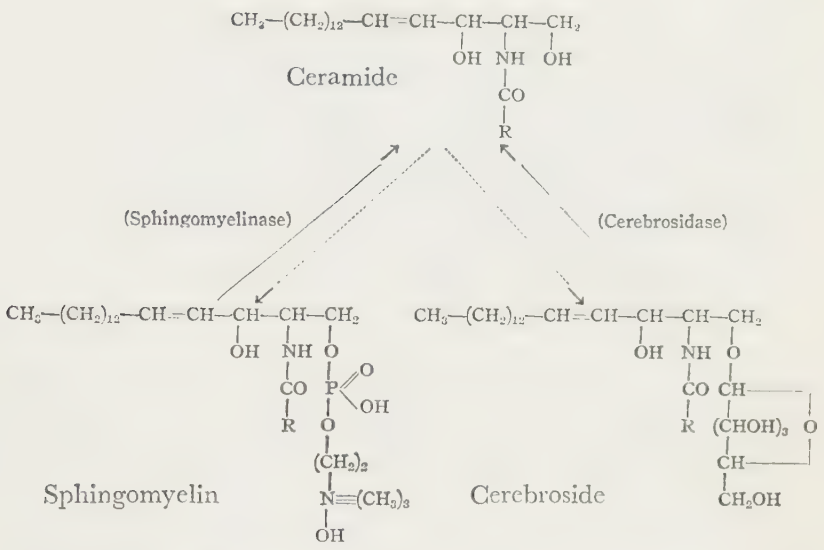


It is possible, of course, that there are other courses *in vivo* concerning the decomposition of sphingomyelin. Especially it is possible that ceramide isrdecomposed further to sphingosine and fatty acid under some other conditions. Nevertheless, the hydrolysis of the type shown above would be very conceivable.

The author is of the opinion that the ester linkage of phosphorylcholine to sphingosine is broken down easily, while the peptidic linkage of fatty acid to sphingosine will not be attacked by the enzymatic action.

Though the isolation of free ceramide was impossible owing to its insufficient amount in the reaction-mixture, the existence of ceramide had been indicated by Thanhauser, Fränkel & Bielshowsky (*8*) and Tropp & Wiederscheim (*9*), who had found this substance as lignocerylsphingosine in liver, lung and spleen. Especially, Thanhauser assumed that ceramide might be an intermediary substance between sphingomyelin and cerebroside, and found that the activators had acted reversely on the enzymatic hydrolysis of both substances. According to Ohno (*10*), the brain of fetus is comparatively rich in ceramide, which diminishes gradually after parturition, being accompanied by the simultaneous increase in sphingomyelins and cerebrosides. Besides, the configuration of sphingomyelin and cerebroside, which have been reported lately by Fujino (*2*) and Nakayama (*11*) of this laboratory, indicates that the difference between the two constitutional formulae exists only in the point that the primary alcohol at the terminal carbon combines either with phosphorylcholine or with galactose.

Taking these observations into consideration, the author is inclined to the hypothesis that sphingomyelins and cerebrosides are so closely related that they can be interchangeable from one to the other in the animal body through the stage of ceramide as follows:



In order to justify this hypotheisis, it must be demonstrated furthermore that cerebroside is hydrolyzed to ceramide by cerebrosidase and that ceramide and phosphorylcholine, or ceramide and galactose are biochemically synthesized to sphingomyelin or to cerebroside by the enzymatic actions. On these subjects the author is intending to continue his further investigation.

SUMMARY

1. Enzymatic hydrolysis of sphingomyelin was attempted.

2. The liberation of phosphorylcholine, choline, and phosphoric acid was almost complete, whereas the amino-group was not liberated.

3. An assumption as to the formation of sphingomyelin and cerebroside was presented.

The athor wishes to thank Prof. Morio Yasuda for his advice and encouragement during this study.

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The Journal of Biochemistry, Vol. 39, No. 1.

STUDIES ON THE INTERACTION BETWEEN PYRIDINE-HEMIN AND HYDROGEN PEROXIDE OR OXYGEN. II. THE FACTORS AFFECTING THE PROCESS OF VERDOHEMOCHROME FORMATION

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(Received for publication, September 11, 1951)

As was postulated in our previous paper (*1*), both hydrogen peroxide and oxygen play essential roles in the process of verdohemochrome formation, in which hydrogen peroxide is expected to evolve from ascorbic acid and oxygen by the catalytic activity of pyridine-hemin. Its activity varies with the change of pH of the reaction medium (*2*).

In the present paper, studies will be made on the effects of pH, the concentrations of ascorbic acid and copper ion upon the velocity of each reaction stage in the formation of verdohemochrome from pyridine-hemin. Subsequently a systematic scheme of the reaction mechnism will be presented on the basis of our experimental results so far obtained.

EXPERIMENTAL METHODS

The used apparatus was the same as described in the previous paper. The changes of the optical densities at 557, 630 and 656 mμ were measured spectrophotometrically along with the reaction proceeds. Applying the constants of the equation described in the previous paper to the optical densities thus observed, each stage of the process under the present experiment could be both independently and quantitatively demonstrated.

A solution of $1.1\times10^{-4}M$ hemin in 20% pyridine was prepared as described in the previous paper and the end concentration of hemin was made up exactly to $1.0\times10^{-4}M$ in each experiment as a starting solution.

RESULTS

Effect of pH—In order to keep the reaction solution at desired pH, 3.0 ml. of pyridine-hemin solution were previously mixed with 0.1 ml.

of NaOH or HCl at a known concentration, and after the addition of 0.2 ml. of $1.5 \times 5.5 \times 10^{-2}$ *M* ascorbic acid neutralized with an equivalent NaOH, aeration was allowed to start. pH of the reaction mixture was determined by the hydrogen double electrode separately with 20% pyridine in place of pyridine-hemin solution and with water in place of ascorbate solution. The influence of hemin upon pH was neglected. As shown by Figs. 1 and 2, the rates of 630-compound and verdohemo-

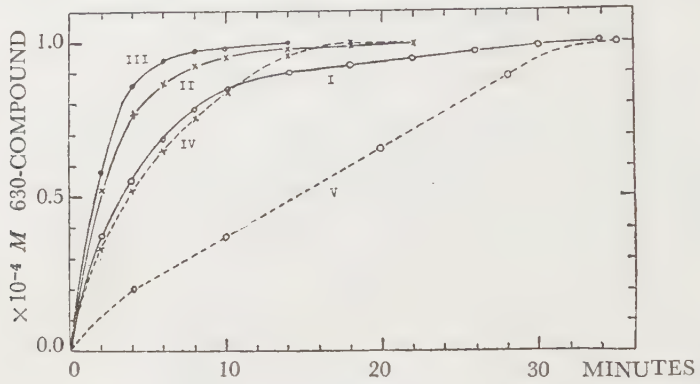


FIG. 1. Changes of the reaction rate on 630-compound formation with the change of pH.

Pyridine-hemin, $1 \times 10^{-}M$. Ascorbic acid, $1 \times 10^{-3}M$. pH: I, 6.88; II, 7.17; III, 9.30; IV, 10.51; V, 11.59.

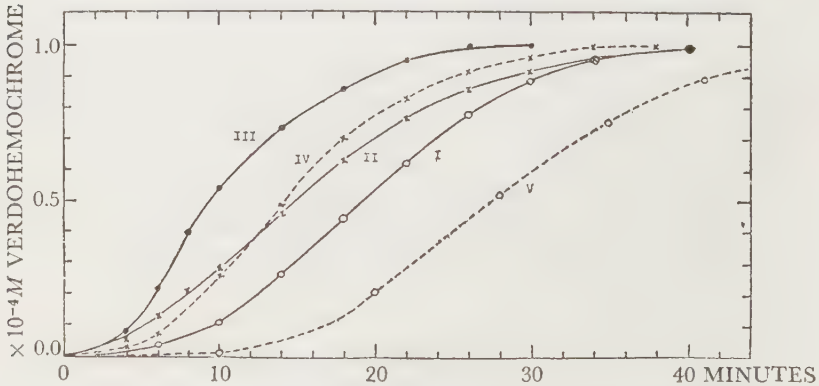


FIG. 2. Changes of the reaction rate on verdohemochrome formation with the change of pH. (Experimental conditions, same as in Fig. 1.)

chrome formation appeared to be affected by changing pH of the medium. An optimal pH condition was secured at about 9.3, nearly corresponding to that of 20% pyridine solution. The reaction velocity decreases with the decrease in pH for the formation of both 630-compound and verdohemochrome, especially of the latter. With the increase of pH above the optimum, the reaction rate decreases: this time especially in the formation of 630-compound.

Effect of the Ascorbate Concentration—(a) The reaction was allowed to start at pH 9.3 with different initial concentrations of ascorbate. The reaction rates in each stage are shown in Fig. 3 and 4. As shown by the figures, the rate of 630-compound formation increases with the increase in ascorbate concentration. With an exceedingly higher

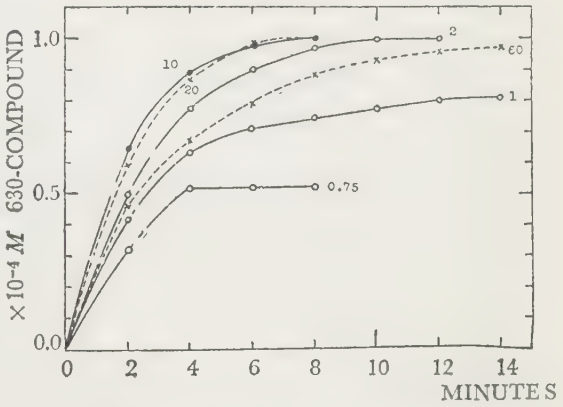


Fig. 3. Reaction rates on 630-compound formation with the different initial concentrations of ascorbic acid.

Pyridine-hemin, 1×10^{-4} *M*; pH, at 9.30. Ascorbic acid is neutralized previously with the equivalent amount of NaOH. Numbers in the figure represent the ascorbic acid concentrations in the unit of $\times10^{-3}$ *M*.

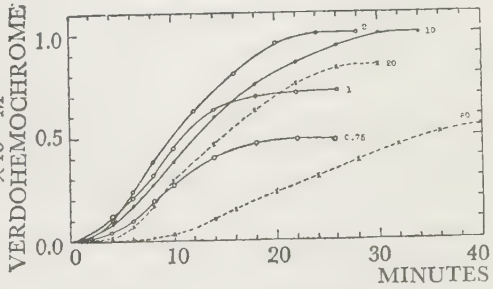


Fig. 4. Reaction rates of verdohemochrome formation with the different initial concentrations of ascorbic acid. (Experimental conditions and the numerical denotations in the figure, are the same as in Fig. 4.)

concentration of the latter, however, the reaction rate decreases. The optimal ascorbate concentration was found to be about 10^{-2} *M*. The inhibitory action of the higher ascorbate concentration is more effective on

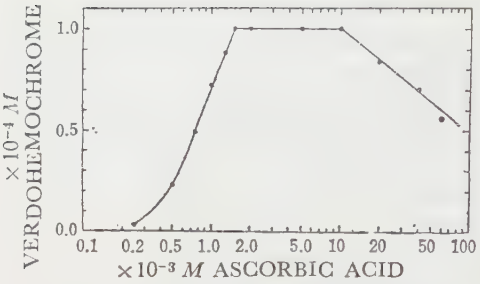


FIG. 5. Relationship between the ascorbate concentration and the amount of verdohemochrome formed at the final state. (derived from Fig. 4.)

the process of verdohemochrome formation from 630-compound. The optimal ascorbate concentration for the latter process was thus found to be much lower than that for the former process. In Fig. 5 is given the relationship between the ascorbate concentration and the amount of verdohemochrome formed at the final state, where the reaction has been completed and its characteristic absorption is finally settled. The maximum verdohemochrome formation was observed at the ascorbate concentration of $1.5 \times 10^{-3} M$.

Further increase in the ascorbate concentration brings about no effect upon the reaction rate, unless the ascorbate concentration becomes to be a considerable excess. This indicates that the reaction of the verdohemochrome formation as the final product proceeds fairly quantitatively and completely by consuming ascorbic acid steadily. On the assumption that the least sufficient concentration of ascorbate for the complete formation of verdohemochrome from pyridine-hemin be $1.5 \times 10^{-3} M$, the necessary molar ratio of hemin to ascorbic acid for the reaction is calculated to be 1:15. With lower concentrations of ascorbate the final amount in the verdohemochrome formation reaches, by no means, its maximum value: the reaction remains in its incomplete state.

If some less powerful $Na_2S_2O_4$, as it is often the case on longer preservation, is added to such an incomplete reaction state, the two absorptions at 557 and 525 mμ will be restored by the reduction, ε_{557} being found to be stronger than ε_{525}, but the absorption in the red will not be changed (Fig. 6). The fact indicates that the final product of the process is always verdohemochrome, but not the 630-compound, *i.e.*, the reaction will not stay in the 630-compound stage even in lower ascorbate concentration. In other words, verdohemochrome is formed from 630-compound by the direct action of molecular oxygen, being independent of the presence of ascorbate. As shown in Figs. 3, 4 and 5, if the ascorbate concentration is held too high, the reaction velocity of each stage decreases and the final amount of verdohemochrome reaches, by no means, its maximum value even by longer aeration. This

is possibly due to an out-of-way decomposition of pyridine-hemin by the excessibly produced H_2O_2 leading to some byproduct formation, which increases with the increase in ascorbate concentration.

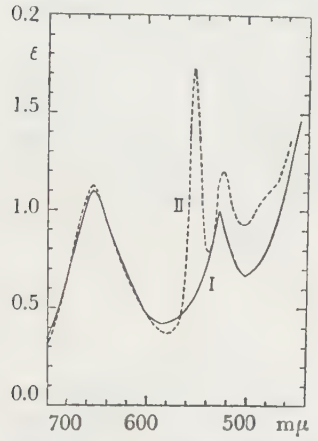


FIG. 6. Absorption curves in the incomplete reaction state.
I. Final reaction state with insufficient amounts of ascorbic acid.
II. After reduction of I with some less powerful $Na_2S_2O_4$.

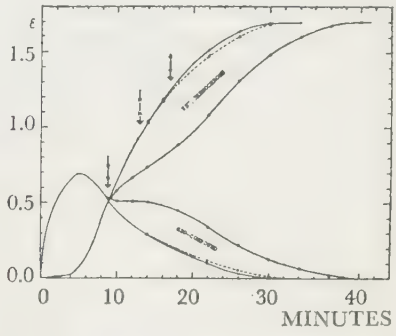


FIG. 7. Effects at the an addition ascorbate during the reaction after the reaction started.

b) *Effect of the Addition of Ascorbate during the Reaction after the Reaction Started*—In the experiments of this series, 3.0 ml. of pyridine-hemin and 0.3 ml. of 0.1 *M* ascorbate were first mixed, aerated and then 5.28 mg. of crystalline ascorbic acid were added to the mixture after 8, 12, and 18 minutes of aeration. The results are summarized in Fig. 7.

When ascorbic acid is added after 18 minutes of aeration, it is found to be almost ineffective to the reaction. The effect of the additional ascorbic acid is evident only in the earlier stage of the aeration. A pronounced effect is observed when the ascorbic acid is added at the 8 minute of the aeration, as shown in Fig. 7. The reaction is evidently depressed by the addition of ascorbate, and the degree of the effect is proportional to its amount added. The rate of verdohemochrome formation increases again after the added ascorbic acid has been used up to a certain extent. The formation of byproducts seems

to increase when larger amounts of ascorbic acid are added, just similarly as obsered in the previous experiments.

Effect of Copper Ion—Copper sulfate solution was used as the source of copper ion. Cu^{++} ion forms a stable complex with pyridine, as well known, giving a beautiful blue solution. The solution of Cu^{+}-pyridine complex is, however, colorless, so that the absorptions at any wavelength will not be changed by the addition of copper ion in so far as a sufficient amount of ascorbate is present. When, however, the added ascorbate has been consumed up through the aeration, there appears in general a sudden and confusing change in the absorption curve with the formation of cupric ions through the oxidation. If such a sudden

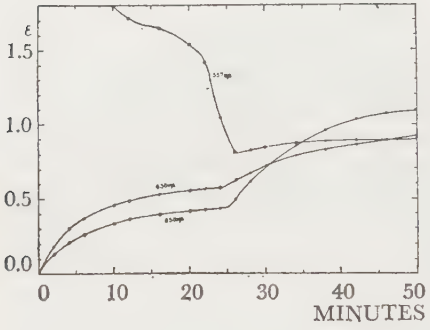


Fig. 8. Sudden confusion occurring in the absorption property by the appearence of cupric pyridine complex, pyridine-hemin, 1×10^{-4} *M*; ascorbic acid, 1×10^{-2} *M*; $CuSO_4$, 1×10^{-3} *M*.

confusion once occurs in the absorption property, it will then be indicated that the reaction remains no more in its ordinary course, the ascorbate being exausted at this point; after that the reaction proceeds with entirely different mode of action without ascorbic acid being present. The above relations are demonstrated in Fig. 8. In the folowing experiments, however, the estimations are mostly given up after the turning point.

a) *The Addtion of Copper Ion at the Starting Point*—The reaction mixtures were prepared as follows and were subsequently aerated: 3.0 ml. of pyridine-hemin solution; 0.1 ml. of $33/300\sim33/6000$ *M* $CuSO_4$ solution; 0.2 ml. of 1.5×10^{-1} *M* ascorbic acid solution.

The results are given in Figs. 9, 10, and 11. The curves in Fig. 9 show the absorption changes of ε_{557} as they were observed. The curves in Figs. 10 and 11 show the actual absorption changes obtained

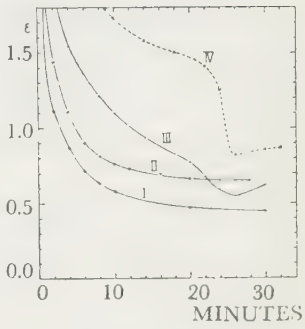


FIG. 9. Retarding in each stage of verdohemochrome formation with the increase in copper ion concentration. a) Decreases in ε_{557} along with the reaction.

Pyridine-hemin, $1\times10^{-4}\,M$; ascorbic acid, $1\times10^{-2}\,M$; $CuSO_4$, I. 1/6000 M, II. 1/3000 M, III. 1/1000 M, and IV. 1/300 M.

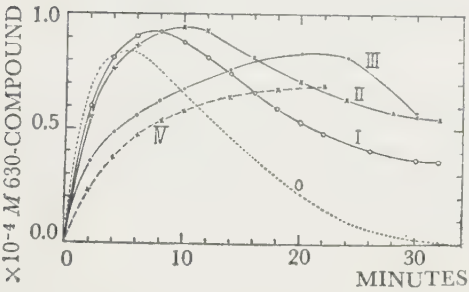
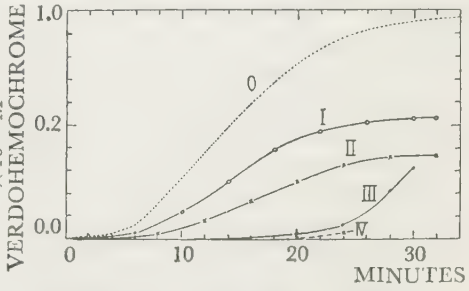


FIG. 10. Retarding in each stage of verdohemochrome formation with the increase in of copper ion concentrations. (b) Changes of the amounts of 630-compound along with the reaction. (Experimental conditions and the denotations in the figure are the same as in Fig. 9, except that O represents in Fig. 10 a control experiment without copper ion.)

FIG. 11. Retarding in each stage of verdohemochrome formation with the increase in copper ion concentrations. (c) Verdohemochrome formation along with the reaction. (Experimental conditions and the denotations in the figure are the same as in Fig. 10.)



by the calculation from the observed ε_{630} and ε_{656} mμ applying the constants of the equation as indicated in the previous paper. The sudden dropping of ε_{557} after the 20 minutes of aeration in Fig. 9, IV, indicates the beginning change of $Fe^{++}\rightarrow Fe^{+++}$ of pyridine-hemin in consequence of the exhaustion of ascorbate. The rates of the conversion of pyridine-hemin and of the formation of both 630-compound and verdohemochrome are, as shown in Figs. 9, 10 and 11,

markedly diminished with the increasing copper ion concentrations, especially on the verdohemochrome formation. At the same time, as indicated demonstratively in Fig. 11, the formation of byproduct is vigorous in the process with copper ion. For the presence of the by-product formation, the following experiments are indicative as shown in Fig. 12.

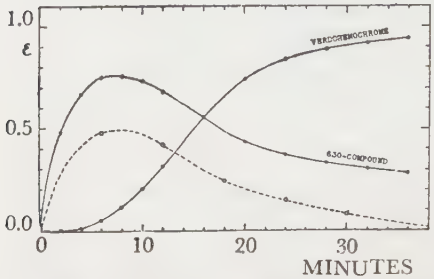


Fig. 12. Amounts of 630-compound actually present at each time along with the reaction.

Pyridine-hemin, 1×10^{-4} *M*; ascorbic acid, 1×10^{-2}*M*; $CuSO_4$ 1/6000*M*.

Dotted line represents the differences between the optical densities at 630 mμ before and after the addition of less powerful $Na_2S_2O_4$ to the reaction solution each time of aeration.

The curve (dotted line) in Fig. 12 was made as follows. The optical densities at 630 mμ were measured before and after the addition of the less powerful $Na_2S_2O_4$ in pulverized state at different aeration times, namely, 6, 12, 18 minutes and so on. The fact should be taken in consideration that 630-compound can be easily reduced even by a less powerful $Na_2S_2O_4$, by which the verdohemochrome scarecely be reduced. Then the differences of ε_{630} before and will after the reduction with $Na_2S_2O_4$ were plotted against the aeration time. With this curve, therefore, the amount of 630-compound actually present at each time is given indirectly, but with certain exactness. According to the curve (dotted line) in Fig. 12, all the 630-compounds should turn out to verdohemochrome in 40 minutes of aeration; nevertheless it follows from the curve in Fig. 12 that the considerable amount of 630-compound is still persisting. The contradiction might be possively caused by the fact that the factors used for the calculation can no more hold under the influences of the contaminating byproducts. A similar consideration should be taken into for the evaluation of the roughly obtained curves of verdohemochrome formation. The results given in Figs. 10 and 11, by no means, express the actual amount of each substance in the system.

(b) *Effect of the Addition of Copper Ion after the Reaction Started*—A slight amount of copper ion was added to the reaction mixture after the reaction started at the point of 4 minutes aeration in one experiment

and of 12 minutes of aeration in another experiment. The results obtained here indicates that the process of the verdohemochrome formation is markedly delayed and the byproduct formation increases immediately on adding copper ion.

DISCUSSION

Process of 630-Compound Formation—A maximum rate of 630-compound formation will be attained at pH 9.3, and if the pH value is kept constant, the reaction rate will increase with the increasing ascorbate concentration within a certain limit. These are possibly due to the increases in H_2O_2 production with the increasing ascorbate concentration. In fact, the O_2 consumption of the system, manometrically estimated, increased with the increase in ascorbate concentration. Two moments thus seem to be involved in the reaction process: primarily, the production of H_2O_2 being catalyzed by pyridine-hemin, and secondarily, the oxidative decomposition of pyridine-hemin by the action of H_2O_2 activated by pyridine-hemin, if not by its own molecule. Further increase in ascorbate resulted, however, unexpectedly in a decrease of the reaction velocity. This fact seems in part to be due to the enhanced peroxidatic activity of pyridine-hemin exerted under the given condition. The enhanced peroxidatic activity of pyridine-hemin will competitively overcome the reaction of 630-compound formation for the decomposition of H_2O_2 produced. From the assumption that 15 molecules of ascorbic acid are necessary for the complete conversion of one molecule of pyridine-hemin into verdohemochrome, it seems to be beyond the doubt that the peroxidatic—presumably also catalatic—decomposition of H_2O_2 has been involved in this reaction process.

Furthermore the rate of 630-compound formation is markedly delayed, when copper ion is added to the system, in spite of the intense increase in O_2 consumption as evidenced by manometric experiments. The fact may be interpreted by the assumption as follows: the addition of copper ion to the reaction system results in the increasing formation of H_2O_2 (*3–5*), and the latter will be converted catalytically either by copper ion or by pyridine-hemin, thus forming a competing system. If the competition is favoured for the activity of copper ion, the successively arising H_2O_2 in the system may be converted more peroxidatively, so that the decomposition of hemin molecule into 630-compound will be depressed more or less. As the matter of fact, the peroxidatic activi-

ty of copper ion is markedly enhanced by the presence of pyridine as evidenced by Tomimura in our laboratory (*6*).

The mode of action of pyridine-hemin in the system seems to be of much interest for the explanation of the reaction mechanism, although it has not yet been sufficiently cleared up at present.

It is important to point out that the reaction leading to 630-compound seems to be accompanied more or less by the existence of the Fe^{+++} state of pyridine-hemin. Thus, inspite of Lemberg's assumption that the reaction starts with the Fe^{++} state of the pyridine-hemin, the final solution of the problem shall be left to the future. At present we are in the feeling that some proper field of the redox-potential seems to be necessary in the medium for the production of 630-compound which is possibly dependent on the state of $Fe^{++} \rightleftarrows Fe^{+++}$ equilibrium. As above mentioned, the formation of 630-compound was delayed in the presence of an excess of ascorbate. It will be in part due to the effect of a high ascorbate concentration which probably makes difficult the $Fe^{++} \rightleftarrows Fe^{+++}$ interchange in the hemin iron.

The effect of pH change may be interpreted also on the same basis. It is also to be pointed out additionally that there exist certain regular relations between the pH value and the redox-potential of pyridine-hemin (*7*) and of ascorbic acid (*8*).

The Process of Verdohemochrome Formation from 630-Compound—As shown by Figs. 4, 6, and 7, ascorbic acid is not only unnecessary for this process, but it even exerts a marked inhibition upon the process. Moreover, as indicated in Fig. 2 of the previous report, verdohemochrome could not be produced form 630-compound by the direct action of H_2O_2. Thus it may be concluded that the process has its relation only to the molecular oxygen. The fact that the verdohemochrome formation is delayed by the excessive presence of ascorbic acid, may be explained by the possibility that in such a condition only the oxidatic and peroxidatic activities of 630-compound dominate in the reaction system, so that the molecular oxygen can be scarecely involved in the process of verdohemochrome formation from 630-compound.

As indicated in Fig. 11, verdohemochrome formation was markedly delayed through the addition of copper ion. In the presence of copper ion, owing to its powerful catalytic activity the oxygen is to be used much for the H_2O_2 production, and the produced H_2O_2 will be then convented peroxidatically, so that the reaction rate of verdohemochrome formation from 630-compound will be relatively diminished as consequence. These relations are indicated demonstratively in Fig. 13: ε_{630} shows a begin-

ning decrease at the point A, at which the ascorbic acid has been exhausted to such a high extent that in accord with the change $Fe^{++} \rightarrow Fe^{+++}$ then taking place in the system the rate of verdohemochrome formation increases suddenly in turn. The ascorbic acid has been completely exhausted at the point B and there appears a profound discordance among the optical properites indicating the arising contamination of Cu^{++}—pyridine complex.

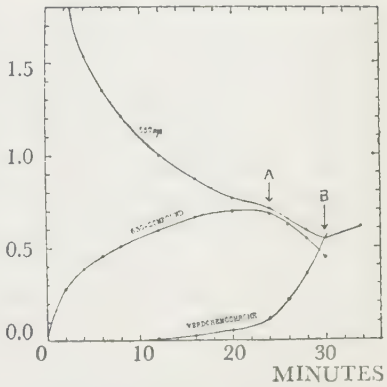


FIG. 13. Sudden increase in verdohemochrome formation after the exhaustion of ascorbic acid.

Pyridine-hemin, 1×10^{-4} *M*; ascorbic acid, 1×10^{-2}*M*; $CuSO_4$, 1×10^{-3} *M*.

As already mentioned, the 630-compound seems to remain in its Fe^{+++} state throughout the reaction. Then the verdohemochrome formation ought to start from the Fe^{+++} state of the 630-compound. The assumption was evidenced through an experiment, in which the 630-compound prepared from pyridine-hemin by the direct action of H_2O_2 in the presence of ascorbic acid, was first reduced by $Na_2S_2O_4$ and then aerated. No change of the optical density has occurred at any wavelength throughout the aeration as far as the hemin was kept in its Fe^{++} state by the action of $Na_2S_2O_4$ until the process of verdohemochrome formation began to take place almost suddenly in accord with the beginning change of $Fe^{++} \rightarrow Fe^{+++}$ as a result of the exhautsion of $Na_2S_2O_4$. It is thus indicative of the fact that the verdohemochrome is formed only from the Fe^{+++} state of the 630-compound, but not from its Fe^{++} state, although the possibility is not wholly excluded that the oxygen is consumed under the given condition merely for the oxidation of $Na_2S_2O_4$, but not for the verdohemochrome formation.

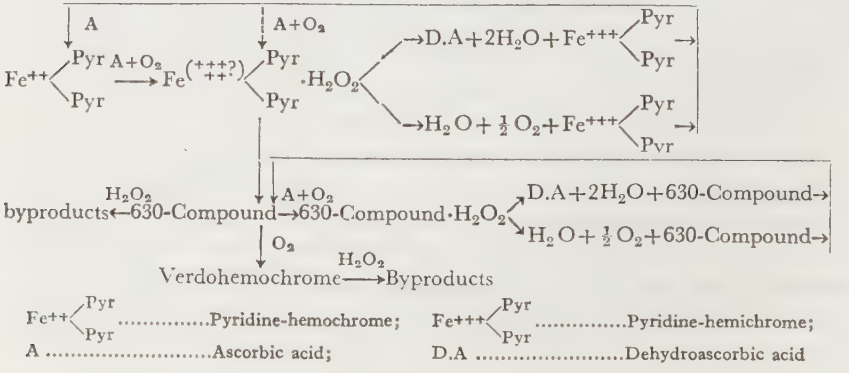
Byproducts Formation—If either the excess of ascorbic acid is present or copper ion is added in the system, there arises a considerable amount of byproducts, seemingly owing to the vigorous production of H_2O_2 in the process. The formation of byproducts may arise certainly from anyone of pyridine-hemin, 630-compound and verdohemochrome. It has been verified, however, that the pyridine-hemin can be converted into the 630-compound almost completely by the action of H_2O_2 in the

presence of ascorbic acid unless H_2O_2 is used in its excess (*9*). Therefore, the byproducts may be formed rather through the breakdown of either 630-compound or verdohemochrome. The following experiments were carried out to solve this problem.

a) Three ml. of verdohemochrome solution prepared by the ordinary procedure was mixed with the additional 10 mg. of ascorbic acid and was subsequently aerated for 30 minutes. In this case the both optical densities of at 656 mμ and 630 mμ were held unchanged throughout the whole course of the aeration. It may now be accepted with no difficulty that the byproduct formed in the presence of the excess ascorbate arises mainly from 630-compound through its decomposition being out of the way.

b) The reaction mixture held under the same condition as above was further mixed with 0.02 ml. of 33/1000 *M* $CuSO_4$ and was subsequently aerated. In this case the both absorption bands at 656 and 630 mμ, especially the former decrease linearly with the length of the time of aeration, thus indicating the possibility of the further decomposition of verdohemochrome leading to some kind of byproducts. Its decomposition, however, is rather too slight to account for the whole amount of byproducts exceedingly formed in the presence of copper ion. The extraordinary high byproduct formation, occurring under the condition in which copper ion has been added at the starting point, will be thus mainly attributed to the abnormal decomposition of the 630-compound.

The Presentation of a Scheme on the Reaction Process—On the basis of the experimental results hitherto obtained, the whole cycle of events in the system of pyridine-hemin, ascorbic acid and oxygen on the formation of verdohemochrome can be summarized as follows.



In conclusion, the reaction between ascorbic acid and oxygen is catalyzed by pyridine-hemin producing H_2O_2, which will be then decomposed by the catalytic activity of pyridine-hemin peroxidatically and catalatically. Thus the pyridine-hemin molecule will be affected in the same course of process, as being oxidized by H_2O_2 activated by pyridine-hemin, if not by its own molecule, and will be converted to 630-compound as the one of the intermediate oxidation products of pyridine-hemin. The nature of the reaction taking place to form 630-compound seems, therefore, to be of a peroxidatic one.

630-compound possesses the catalytic activity similar to its precursor, but rather as the peroxidatic. The compound is relatively stable against H_2O_2, but is easily converted into verdohemochrome by molecular oxygen. The conversion is therefore the autoxidation of 630-compound by oxygen which is activated by its own molecule. The fact that 630-compound reacts only with molecular oxygen will afford an important suggestion upon the chemical structure of its molecule.

If H_2O_2 is produced in excess in any way, the formation of the by-products will more or less increase, being accompanied by the ordinary products.

SUMMARY

The effects of pH, and the concentration of ascorbic acid and copper ion on the process of verdohemochrome formation were studied in its each stage by means of spectrophotometric method. The results obtained are conclusively summarized as follows.

1. Both the decomposition of pyridine-hemin into 630-compound and the conversion of 630-compound into verdohemochrome are the processes of the autocatalytic oxidations in their nature, connected with their catalytic activities.

2. A certain reductant can be indispensable for the 630-compound formation. It is difficult, however, to determine whether the Fe^{++} or the Fe^{+++} state of pyridine-hemin is more responsible for the process.

3. Verdohemochrome can be produced from the Fe^{+++} state of 630-compound by the direct action of molecular oxygen without any reductant.

4. If H_2O_2 is produced excessively, for example, by higher concentration of ascorbic acid or by the addition of copper ion, the by-product formation will increase through a side reaction.

5. On the basis of the experimental evidences hitherto obtained including those from our previous information a scheme on the re-

action processes in the pyridine-hemin—ascorbic acid—oxygen system is hypothetically presented.

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BIOCHEMICAL STUDIES ON HYALURONIC ACID AND HYALURONIDASE. III. ACTIVATION OF SKIN HYALURONIDASE BY THE ACTION OF BLOOD CORPUSCLES

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It seems to be generally conceded at present that hyaluronidase in skin can exist either in bound or free state (*1*, *2*) and the fact that the activity of hyaluronidase is increased during the prosess of inflammation (*3*) or the autolysis of skin *in vitro* (*4*) has been interpreted as being due to the conversion from bound to free state of the enzyme. It should, however, be pointed out that the increase of hyaluronidase activity in the inflammatory process can be provoked even by a slight injury of skin, and moreover, it occurs by far makedly and quickly compared with that caused by the autolysis of skin *in vitro*. These facts seem to suggest that different mechanisms may underly the phenomena of the increase in hyaluronidase activity in these two cases.

In the present paper it will be shown that the appearance of hyaluronidase activity in autolysing skin is strongly accelerated by the addition of small quantities of whole blood or blood corpuscles. It is, therefore, envisaged that the difference in mechanism between the two cases in question may lie, at least partly, in the participation of blood corpuscles in the process occurring in the inflammation of skin.

EXPERIMENTAL

Hairs were removed from the side abdominal regions of healthy guinea pigs which were then killed by bleeding. The skin was immediatly cut off and minced under aseptic condition. 1 g. of the minced skin was suspended in 5 ml. of *M*/15 phosphate buffer (pH 7.5) containing 0.9% NaCl and various quantities of blood or blood corpuscles, and with the addition of toluene it was left for autolysis at 37° for 16 hours. Autolysates were filtered and the enzymic activities of filtrates were

measured by viscosimetry using Ostwald's viscosimeter. For this purpose, 0.5 ml. of the filtrate was added to 2 ml. of the substrate solution (0.6 mg./ml.) which was dissolved in acetate buffer (pH 6). The final concentration of the buffer was 0.1 N containing 0.9% NaCl. The viscosity of the mixture was followed at 37°, and the time required for the reading to reach the mean value of the initial and final readings was determined. The length of this time, which will be referred to as "half life time" (HLT) in the following, was used as the measure of the enzyme activity.

Results obtained are shown in Tables I and II.

TABLE I

Activation of Skin Hyaluronidase by Blood

Exp. No.	Concn. of whole blood	Skin added	Relative viscosity after				HLT
			0 *min.*	5 *min.*	10 *min.*	20 *min.*	
1	1/640	+		1.89	1.88	1.85	+
2	1/320	+		1.71	1.58	1.43	18′ 20″
3	1/160	+		1.93	1.93	1.93	—
4	1/80	+	1.905	1.91	1.91	1.91	—
K	0	+		1.97	1.96	1.94	—
B	1/40	—		1.96	1.96	1.94	—

K: Autolysate of the minced skin without addition of whole blood.

B: Autolysate of whole blood without addition of minced skin.

Concentration of whole blood: The ratio of dilution of whole blood before addition of minced skin.

Relative viscosity at 0 minute is the value obtained with substrate solution, to which was added the same quantity of distilled water in place of the filtrate or autolysed skin.

HLT: —, the viscosity of the reaction mixture remained unchanged; +, the change in viscosity occurred, but with such a long HLT that it could not be measured.

TABLE II

Activation of Skin Hyaluronidase by Blood Corpuscles

Exp. No.	Concn. of blood corpuscles	Skin added	Relative viscosity after				HLT
			0 *min.*	5 *min.*	10 *min.*	25 *min.*	
2	1/40	+		1.73	1.60	1.46	15′
3	1/80	+		1.90	1.85	1.77	45′
5	1/320	+	2.00	1.98	1.96	1.93	+
K	0	+		2.00	2.00	2.00	—
B	1/20	—		1.95	1.95	1.95	—

In this case, blood was cooled immediately after bleeding and blood corpuscles were centrifuged out, washed twice with sterilized saline and finally suspended in saline of original volume.

From the results presented in these tables it can be seen that the skin autolysed in the presence of whole blood or blood corpuscles showed by far higher hyaluronidase activities than that autolyzed without addition of these substances. Experiments not reproduced here have revealed that blood serum is totally devoid of such an ability of activation. These results show convincingly that blood corpuscles play an essential role in the activation of the enzyme.

It should be remarked that the autolysates of skin used in these experiments were not absolutely free from bacterial contamination; from the samples we could cultivate some strains of *Staphylococcus albus*, *Staphylococcus aureus*, *Esherichia coli*, *Proteus vulgaris*, *etc.* It was, however, confirmed that the distribution of different bacteria in different samples had no relation to the enzyme activity of the autolysate, and moreover, the cultures of isolated bacteria were found to be without any action upon hyaluronate. It is, therefore, impossible to consider that the contaminated micro-organisms in the autolysate would have contributed to the phenomenon of the activation of hyaluronidase.

SUMMARY

1. The activity of hyaluronidase in autolyzing skin was found to be remarkably enhanced by the addition of whole blood.

2. The effective part of whole blood was found to be blood corpuscles. Blood serum showed no effect.

3. There seems to be much resemblance between the activation of enzyme here stated and the activation of skin hyaluronidase in the case of inflammatory process.

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EFFECT OF DENATURATION UPON ANTIGENIC REACTIVITY OF PROTEIN MONOLAYERS*

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Since the works of Raileigh (*1*), Langmuir (*2*), Adam (*3*), Rideal (*4*) and others, the study of surface film has much contributed to the problems of fatty acids, sterins and other surface active substances. Especially the surface chemical studies of protein by Langmuir (*5*), Rideal (*6*), Gorter (*7*) and recently by Dervichian (*8*), Bull (*9*), Rothen (*10*) and others have yielded a great deal of illuminating results about this complex biological substance.

The protein monolayer, the thickness of which is calculated and determined (*11*) to be 8–10 Å, is considered to be an ideal type of denaturated protein from various experiments (*12*). In such a monolayer the antigenic reactivity of protein is retained undestroyed according to the experiments of Marrack (*13*), Rothen and Landsteiner (*14*), while in usual experiments denaturation is always accompanied by marked decrease or disappearance of the antigenic reactivity of protein (*15*). This fact was explained by the theory that antigenic reactivity of protein is located upon small spots of protein molecules which are not destroyed by film spreading. This theory is based upon the famous works of artificial antigen-antibody reaction of Landsteiner (*15*).

The question whether the decrease of antigenic reactivity by denaturation is merely due to the destruction of active spots of protein molecule or the lack of antigen-antibody reaction by deformation of antigen-molecule is of great interest. This can be solved by comparing the antigenic reactivities of surface films of native and denatured proteins. This problem was partly worked out by Rothen and Landsteiner (*14*), who observed no difference in the case of heat denatured egg albumin, while in horse globulin less antibody was adsorbed by heat denatured protein film. The author wanted to study this problem further, with several denaturating agents such as heat, acid, and alkali, with or with-

* This report was presented before on the 66th Meeting of Kanto District of the Japanese Biochemical Society at Tokyo, 11th November 1950.

out combination of surface spreading and examined the effect of denaturation upon antignic reactivity of protein monolayers. The experiments were carried out in 3 cases:

(I) Protein was denatured, and then spread upon water surface;
(II) Protein was spread and denatured at the asme time;
(III) Protein was spread and then denatured.

In (II) and (III) cases, the active groups of antigen molecule can react directly with denaturating agents, the effect of which can be shown clearly, since upon surface film the reactive groups are bare compared with those in native protein molecules.

EXPERIMENTAL

Immunization—As an antigen, crystallina serum albumin was used. This was fractionated with Na_2SO_4 and purified electrophoretically. 1 % solution of this albumin was injected to five rabbits three times a week. After 15 injections the blood was collected and serum was separated and stored in an ice box with thymol.

Spreading of Film—The spreading of Ba stearate and of protein was carried out according to Langmuir and Blodget (*16*). Ba stearate film was made upon the solution slightly buffered with carbonate at pH 7. Horse serum albumin film was spread upon the buffered solution at pH 4.9.

Deposition of Film—A clean, optically flatt stainless steel plate was first dipped into the solution covered with Ba stearate film, and raised solwly. This plate with Ba stearate monomolecular film was then dipped into the solution covered with protein monolaoer, and raised again. In this case the plate was covered with one double layer of protein. If one single layer of protein was wanted, the plate was washed with water while wet. When only one down-trip layer was needed, the metal plate was dipped first into the clean buffer solution, then protein monolayer was spread, and finally the plate was raised. In each case the film thickness was measured by the method cited below immediately to check whether the film is completely spread or not.

Reaction with Immune Serum—After the plate was dried a drop of immune serum was placed upon the plate, allowed to react for 5 minutes, and washed first with 0.85% NaCl solution, then with water. The increase of adsorbed antibody thickness was measured.

*Measurement of Film Thickness**—Film thickness was measured by

* The details of procedures will be described elsewhere.

the method of Tronstad (*17*), Winterbottom (*18*), and Rothen (*19*). The change of ellipticity after reflexion from the stainless steel covered with stearate film was measured as a standard for the calibration of the unknown film thickness of protein, since the refractive index of stearate and protein is nearly the same, and the change of ellipticity depends solely upon the angle of incidence, adsorption and refractive indice of metal and refractive index of covered material. In this case, using a 18-chrome stainless steel plate with incidence angle of 75°, one double layer of Ba stearate produced the change of ellipticity by about 2°. The accuracy of this instrument ranges 4–6 Å per 0.1°.

RESULTS

I. Protein was denatured, spread upon water surface and its antigenic reactivity was examined.

(a) *Acid Denatruation*—One % solution of crystalline horse serum albumin was mixed with an equal volume of 0.1 *N* HCl, allowed to stand at 37° over night, and neutralized with 0.1 *N* NaOH. This solution was diluted one hundred times to examine its antigenic reactivity by precipitin reaction, or diluted to 0.05% solution to spread upon

TABLE I

The Precipitin Reaction of Acid Denatured Horse Serum Alubimin and Anti-Horse Serum Albumin Serum

	Anti-horse serum albumin serum				
Dilution of antigen	10	100	1000	10000	100000
Acid denatured horse albumin	+	+	±	−	−
Normal horse serum albumin	+	+	+	+	−

TABLE II

The Monomolecular Reaction of Acid Denatured Horse Serum Albumin and Anti-Horse Serum Albumin Serum

	Thickness increase of adsorbed antibody (Å)			Thickness increase of normal serum (Å)	
Acid denatured horse albumin	55	50	50	15	10
Normal horse serum albumin	60	55		15	15

water surface for the examination of antigenic reactivity of denatured protein monolayers. In each case, normal rabbit serum was used as the control. The increase of antibody thickness is shown in Tables I and II.

(b) *Alkali Denaturation* **1**—Instead of 0.1 N HCl in the previous experiment, 0.1 N NaOH was used as a denaturating agent. Other procedures were the same. The retults are given in Tables III and IV.

TABLE III

The Precipitin Reaction of Alkali Denatured Horse Serum Albumin **1** *and Anti-Horse Serum Albumin Serum*

	Anti-horse serum albumin serum				
Dilution of antigen	10	100	1000	10000	100000
Alkali denatured albumin **1**	+	+	−	−	−
Normal horse serum albumin	+	+	+	+	−

TABLE IV

The Monomolecular Reaction of Alkali Denatured Horse Serum Albumin **1** *and Anti-Horse Serum Albumin Serum*

	Thickness increase of adsorbed antibody (Å)					Control (Å)
Alkali denatured albumin **1**	45	45	60	50	50	10
Normal horse serum albumin	60	50				

(c) *Alkali Denaturation* **2**—In this experiment, protein was treated with 0.1 N NaOH for a week at 37°. Other procedures were the same. The results are shown in Tables V and VI.

TABLE V

The Precipitin Reaction of Alkali Denatured Horse Serum Albumin **2** *and Anti-Horse Serum Albumin Serum*

	Anti-horse serum albumin serum				
Dilution of antigen	10	100	1000	10000	100000
Alkali denatured albumin **2**	−	−	−	−	−
Normal horse serum albumin	+	+	+	+	+

TABLE VI

The Monomolecular Reaction of Alkali Denatured Horse Serum Albumin **2** *and Anti-Horse Serum Albumin Serum*

	Thickness increase of adsorbed antibody (Å)				Control (Å)	
Alkali denatured albumin **2**	25	0	4	10	15	10
Normal horse serum albumin	35	30			10	10

(d) *Heat Denaturation*—In order to prevent coagulation which renders the spreading of film impossible, 1 ml. of 1 % crystalline horse serum albumin solution was mixed with 7 ml. of 1 % Na-acetate and 12 ml. of propanol, heated under a reflux condensor for one hour, and then submitted to spreading on water surface according to Staellberg (*20*). In this experiment, after dialysis of this solution to remove propanol, the complete coagulation occurred, so it was impossible to carry out precipitin reaction. The increase of antibody thickness adsorbed by this heat denatured albumin monolayer is given in Table VII.

TABLE VII

The Monomolecular Reaction of Heat Denatured Horse Serum Albumin and Anti-Horse Serum Albumin Serum

	Thickness increase of adsorbed antibody (Å)						Control (Å)
Heat denatured albumin	35	30	5	30	40	30	10
Normal horse serum albumin	35	30					15

II. Protein was denatured and spread at the same time, then its antigenic reactivity was examined to study the effect of pH of underlying water upon antigenic properties of protein monolayer.

Protein was spread upon the surface of water at various pH. The results of monomolecular antigen-antibody reaction are shown in Table VIII together with those of normal rabbit serum.

III. Monomolecular antigenic film was treated with denaturating agents.

(a) *Acid and Alkali*—Monomolecular antigenic film on metal surface was treated with acid and alkali for 5 minutes except in the

TABLE VIII

The Effect of pH of Underlying Water upon Antigenic Reactivity of Horse Serum Albumin Monolayer

pH	Thickness increase of adsorbed antibody (Å)						Control (Å)	
1	30	25					10	10
2	25	25						
5	30	35	45				5	10
11	30	25	25					
12	5	−5	5	10			10	10
13	0	10	10	0	5	10	15	10

case of pH 1 (0.1 *N* HCl for 20 seconds), where the metal surface was destroyed promptly. The results are given in Table IX.

TABLE IX

The Action of Acid and Alkali agsinst Horse Serum Albumin Monolayer on Monolayer on Metal Surface

pH	Thickness increase of adsorbed antibody (Å)			Control (Å)	
1	20	35		10	5
2	25	25		10	10
3	25	40			
6	20	30			
8	25	30		10	5
10	15	10			
11	5	15	20		
12	10	10	0		
13	15	10		10	10

(b) *Heat*—Monomolecular antigenic film on metal surface was heated at 100° (with or without water vapor), 150° or 200° for one hour, The results are shown in Table X.

TABLE X

The Heat Action against Horse Serum Albumin Monolayer upon Metal Surface

Temperature	Thickness increase of adsorbed antibody (Å)					Control (Å)	
Room temp.	20	30				10	5
100° (dry)	25	20				15	5
100° (vapor)	25	20	25			5	10
150°	30	25	25			10	15
200°	20	20	20	20	25	10	15

DISCUSSION

The difference of antibody thickness increase between native and denatured antigen (horse serum albumin) by monomolecular reaction was not observed except in the experiment of alkali denaturation **2**, while antigen-antibody reaction of denatured antigen with precipitin was much reduced compared with that of native antigen. Thus the results of Rothen and Landsteiner were confirmed and extended acid or alkali to denaturation by using crystalline serum albumin as an antigen. Then it might be deduced (at least in the case of egg and horse serum albumin) that denaturating agents such as acid, alkali, or heat do not destroy the active centers of antigen molecule, the decrease or the abolishment of antigenic reactivity of protein by precipitin reaction due to the aggregation or to the deformation of antigen molecules by which the approach of antigen to antibody molecules is strongly hampered. In the alkali denaturation **2**, however, the antigenic reactivity of horse serum albumin was also lost on metal surface. This phenomenon may be attributed either to the hydrolysis of protein molecules into small fragments by the action of alkali, though it could be spread upon water surface, or to the destruction of the active centers of antigen molecules by alkali, which is shown in experiments II and III. The author can not decide between these two possibilities, but it might be said at least that alkali acts on protein in two steps, the first being denaturation in narrower sense and the second hydrolysis or destruction of active groups.

In experiments II and III, where denaturation could occur directly at the active centers of antigenic molecules, it was shown that alkali was

very effective while heat and acid gave less influence. The effectiveness of alkali on antigenic reactivity has been recognized since the beginning of immunochemistry. The reason of this effect is in part explained by such experiments that alkali acts more drastically than heat and acid concerning the destruction of active groups of antigen molecules. In experiment I, however, alkali was also more effective than acid (and perhaps heat) in regards to precipitin reaction, though the active centers of antigen molecules were not destroyed in each case. The effectiveness in the latter case must be further investigated.

SUMMARY

The effect of denaturation (acid, alkali, and heat) on antigenic reactivity of crystalline horse serum albumin monolayer was examined.

1. Without secondary effect, the antigenic reactivity of monomolecular film of denatured protein was the same as that of native one, namely the decrease of antigenic reactivity by denaturation is not due to the destruction of active groups of antigen molecules.

2. In alkali denaturation the reaction seemed to proceed in two steps, the first being denaturation in narrower sense, namely, aggregation or deformation of antigen molecules without destruction of antigenic active groups, and the second being hydrolysis or destruction of active groups.

3. The treatment of antigenic film upon water or metal surface with acid, alkali and heat entailed the direct attack of active groups by denaturating agents. From the results cited above, the author can assume that the active groups of horse serum albumin are weak against alkali, while they are resistant against heat as well as acid. Thus, the author can explain, at least partly, the stronger effect of alkali on the destruction antigenic reactivity in comparison with those of acid and heat.

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PYRUVATE AND α-KETOGLUTARATE IN BLOOD AND URINE. II. EFFECTS OF THIAMINE- AND RIBOFLAVIN-DEFICIENCY

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In the preceding paper (*1*) Shimizu reported that the injection of thiamine or riboflavin showed some effects on the pyruvate or α-ketoglutarate concentration in human blood, especially on those after exercise. In the present paper the authors wish to report the effects of thiamine- or riboflavin-deficiency on the pyruvate and α-ketoglutarate values in blood and urine of rats.

EXPERIMENTAL

Rats, weighing about 150 g. at the beginning of the experiment, were used. They were kept in individual, double screen bottom cages, to prevent coprophagy.

For the experiment of thiamine-deficiency, the following basal diet was supplied *ad libitum*: Purified casein 72 g.; starch 272 g.; soy-bean oil 20 g.; salt mixture (Steenbock no. 32) 16 g.; liver oil 8 g.; water 500 g. mixed well and heated in a water-bath to become half-solid paste. This basal diet was found to be free of thiamine. The vitamin B_2 group was supplied as an aqueous solution containing 4.0 mg. of riboflavin, 5.0 mg. of pyridoxine, 20.0 mg. of niacin, 20.0 mg. of calcium pantothenate, and 1.0 g. of choline hydrochloride in the total volume of 10 ml. Rats, weighing 100-200 g., and over 200 g., were administered daily with 0.10 ml., and 0.15 ml. of this solution, *per os*, respectively. Prior to the deficiency experiment, each rat had received daily 0.15 ml. of 40 mg./dl. thiamine soultion for 8—16 days.

For the experiment of riboflavin-deficiency, the same basal diet as in the above experiment was given to some rats, except that the casein used was purified further by alcohol. For the other greater part of the rats, 280 g. of cane sugar and 16 g. of agar-agar were used instead of the starch of the above basal diet. Both of these diets contained no measura-

ble riboflavin. Each of these basal diets gave almost the same effects on rats for all the experiments on riboflavin-deficiency. The vitamin B group except riboflavin was supplied by an aqueous solution of 8.0 mg. of thiamine, 10.0 mg. of pyridoxine, 40.0 mg. of niacin, and 40.0 mg. of pantothenate in the total volume of 10 ml. Each rat received daily 0.10 ml. of this solution *per os*. Besides it, prior to the experiment, each rat had received daily 100 μg. of riboflavin dissolved in 0.25 ml. of water *per os* for 12–13 days. To prevent the biosynthesis of riboflavin in intestine, daily 10 mg. of sulfaguanidine suspended in 0.15 ml. of water were given *per os* at the proper time.

Daily, urine was collected. Blood sample was obtained by decapitation. The methods for estimating pyruvate and α-ketoglutarate were the same as those in the preceding report (*1*), and lactate was estimated by the method of Barker and Summerson (*3*).

RESULT

Urinary Excretion of Pyruvate and α-Ketoglutarate through the Course of Thiamine-Deficiency—Four rats were fed on the thiamine-deficient diet. The whole course of the changes of their body weight, pulse frequency, food intake and the daily excretion of pyruvate and α-ketoglutarate in the urine is shown in Fig. 1. In one of 4 rats urinary pyruvate

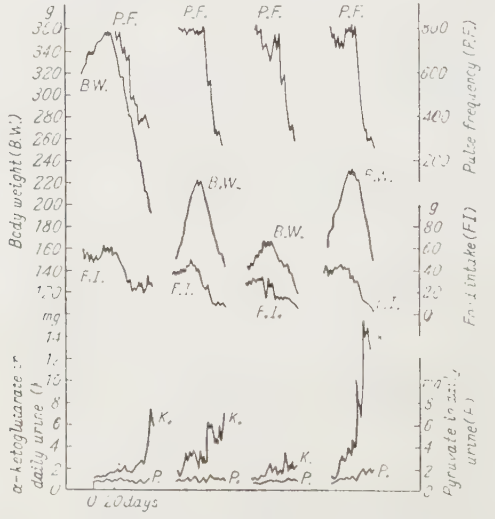

Fig. 1. Pyruvate, lactate and α-ketoglutarate in urine through the course of thiamine-deficiency (↓ indicates the beginning of the thiamine-deficient diet.)

increased markedly, but in the other three rats the increase of pyruvate excretion was not so great. On the other hand the increase of the daily excretion of α-ketoglutarate occurred earlier and more markedly, namely, urinary α-ketoglutarate began to increase already a few days after the feeding with the thiamine-deficient diet was started and before the changes of the increasing rate of body weight, food intake and pulse frequency appeared. The increase went on further as the thiamine-deficiency proceeded.

Pyruvate, α-Ketoglutarate and Lactate in Blood and Urine at Various Stages of Thiamine-Deficiency—Pyruvate, α-ketoglutarate and lactate in blood and urine were determined at the following 6 stages: (a) During the period in which thiamine was given; (b) at the beginning of the decrease of the body weight (on the 14th—16th day from the beginning of the deficient diet); (c) at the period of a marked decrease in body weight (on the 22nd—23rd day); (d) at the period when bradycardia appeared (on the 29th—32nd day); (e) at the final period when paralysis appeared (on the 45th—58th day); (f) after the recovery from the deficiency symptoms by the administration of the daily 60 μg. of thiamine for 5—10 days after they had been fed on the deficient diet for 31–40 days and bradycardia had appeared.

The average values of pyruvate, α-ketoglutarate, and lactate in the blood and urine of 2–4 rats at each stage are shown in Table I. Pyruvate in blood and urine began to increase after 2 weeks from the beginning, and at the final phase of deficiency the values were about 3–4 times as much as those at the beginning. α-Ketoglutarate in blood and urine showed a distinct increase already after 2 weeks from the beginning, and especially, the urinary excretion was about twice as much as that at the beginning. There after the values increased further and at the final phase the concentration in blood was about 5 times as much as that at the beginning, and the urinary excretion was over 10 times as much as that at the beginning. Lactate in blood increased also in the final phase, but the degree of increase was far less than that of pyruvate, and so the ratio of lactate to pyruvate decreased markedly during the whole course of the deficiency. When the rats were recovered from the deficiency symptoms by the administration of thiamine, these values of pyruvate, α-ketoglutarate, and lactate returned to the normal values.

Isolation and Identification of α-Ketoglutarate as 2,4-Dinitrophenylhydrazone from the Urine of Thiamine-Deficient Rats—To confirm that the increased α-ketoglutarate value in the urine of the thiamine-deficient rats

TABLE I

Pyruvate, Lactate and α-Ketoglutarate in Blood and Urine of Thiamine-Deficient and Control Rats

		Normal rats	Thiamine-deficient rats				Recovered rats
		Body wt. increase (60 μg. of thiamine given,)	Body wt. begins to decrease	Marked body wt. decrease	Bradycardia appears	Final phase of thiamine deficiency	After bradycardia appeared. (60μg. of thiamine given for 5–10 days:)
Numbers of rats		4	2	3	3	3	3
Days maintained on thiamine-deficient diet			13–15	21–22	28–30	44–57	31–40
Blood	Pyruvate *mg./dl.*	1.21	1.48	3.99	4.29	5.21	2.12
	α-Ketoglutarate *mg./dl.*	0.52	0.74	2.37	2.68	2.69	0.46
	Lactate *mg./dl.*	20.0	17.0	36.3	21.9	42.3	19.2
	Lactate / Pyruvate	16.8	11.5	9.1	5.1	8.1	9.1
24 hrs. urine*	Pyruvate *mg.*	0.26	0.28	0.51	0.63	0.80	0.23
	α-Ketoglutarate *mg.*	0.44	1.04	3.23	3.30	5.27	0.46
	Lactate *mg.*	1.11	1.13	1.03	1.50	1.17	1.03

* The average value of 2–7 days in each rat.

was truly due to α-ketoglutarate, the following experiment was carried out. 157 ml. of urine were collected from the thiamine-deficient rats. The measured α-ketoglutarate content of this total urine was 113 mg. The solution of 2,4-dinitrophenylhydrazine in 2 *N*-HCl was added to the urine, and the mixture was placed at 25° for 30 minutes. The precipitates formed were collected on a filter paper and transferred completely to a centrifuge tube with about 20 ml. of 2% Na_2CO_3 solution as solvent. The dissolved solution of the precipitates was acidified with 0.5 *N*-HCl, and the precipitates formed were centrifuged, washed thrice with

water, and dissolved in 40 ml. of ethyl acetate. After the solution was filtered, ethyl acetate was evaporated in a vacuum desiccator. The residue was treated, 3 times as above, each time with Na_2CO_3-solution, hydrochloric acid and water. The crystals thus obtained were washed with 1 ml. of xylene and dried completely. 189 mg. of the crystals were obtained, whose melting point was 223°, being almost equal to the melting point of 2,4-dinitrophenylhydrazone of synthesized α-ketoglutaric acid (224°), and showing almost the same melting point (222°) by the mixed melting point test. The quantity of the crystals obtained corresponds to 75% of the α-ketoglutarate expected to be present in the urine. Thus it was proved that most of the increased compound measured as α-ketoglutarate in the urine of thiamine-deficient rats was definitely α-ketoglutarate.

Urinary Excretion of Pyruvate, α-Ketoglutarate and Lactate through the Course of Riboflavin-Deficiency—Six rats were fed on the riboflavin-deficient diet and compared with 3 control rats fed on the complete diet. For 62 days from the beginning the vitamin B group except riboflavin was supplied daily as an aqueous solution of 40 μg. of thiamine, 50 μg. of pyridoxine, 200 μg. of niacin, and 200 μg. of pantothenate. The increase of the body weight of each rat stopped soon after the beginning of the riboflavin-deficient diet, and the skin symptoms appeared after 15–19 days. At this stage the apparent increases in pyruvate, α-ketoglutarate and lactate were observed. In one of the 6 rats the deficiency symptoms showed rapid progress and death came after 36 days. The dosage of thiamine was thought to be insufficient in the above diet, though no symptoms of thiamine-deficiency were yet observed in all the rats (*2*). Therefore, the daily dose of the vitamin B group except riboflavin was duplicated for the other 5 rats, by which the daily excretion of pyruvate, α-ketoglutarate and lactate was observed to approach the normal value. In these rats the body weight began to show the increase and the skin symptoms began to disappear after 23–55 days from the beginning. The administration of sulfaguanidine brought about again the decrease of body weight and the skin symptoms.

As time went on, the body weight continued to decrease and the symptoms of riboflavin deficiency became so severe that death come after 105–110 days in 2 rats, but during the course of this stage the daily excretion of three acids was in the normal range. The whole course of one of these rats (no. 3) is shown in Fig. 2. Three of the 5 rats were cured from the deficiency symptoms by the administration of the daily 100 μg. of riboflavin, and also in this phase the daily excretion of the three acids

was in the normal range. An example of the whole course of a rat (no. 6) is shown in Fig. 2.

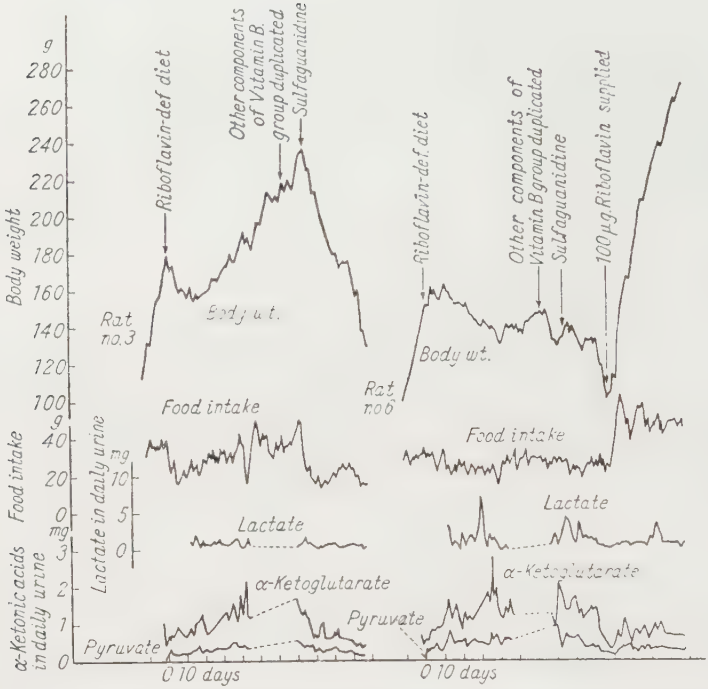

FIG. 2. Pyruvate, lactate and α-ketoglutarate in urine through the course of riboflavin-deficiency.

Pyruvate, α-Ketoglutarate and Lactate in Blood and Urine at Various Stages of Riboflavin-Deficiency—Nine rats were fed on the complete diet containing 100 μg. of riboflavin per day for 12–13 days. The vitamin B group except riboflavin was supplied in the duplicated quantity in the above experiment. The daily excretion of pyruvate, α-ketoglutarate, and lactate was observed for 3 days and after that the concentration of these acids in their blood was estimated with 3 of the 9 rats. The other 6 rats were fed on the riboflavin-deficient diet, and on the 6th–13th day, when the body weight began to decrease, the daily excretion and the blood content of the three acids were measured with the 3 rats. The remaining 3 rats were fed on the riboflavin-deficient diet further for 38–

45 days and when their skin symptoms were marked, the same observation was carried out. As a control, 3 rats were fed on the complete diet for 38–46 days, and also the same observation was carried out with them.

The result is shown in Table II. The quantities of pyruvate and α-ketoglutarate in blood and urine showed no difference between the above groups. The lactate content showed a marked fluctuation in each rat and in each phase, and no definite tendency toward increase was observed in it.

TABLE III

Pyruvate, Lactate and α-Ketoglutarate in Blood and Urine of Riboflavin-Deficient and Control Rats

		Normal rats	Riboflavin-deficient rats		Control rats
		Body wt. increase (100μg. riboflavin given)	Body wt. begins to decrease	Skin symptoms appear markedly	100μg. of riboflavin given for 38–46 days
Number of rats		3	3	3	3
Days maintained on riboflavin-deficient diet			5–12	38–45	
Blood	Pyruvate *mg./dl.*	1.10	1.10	1.09	1.13
	α-Ketoglutarate *mg./dl.*	0.59	0.77	0.72	0.72
	Lactate *mg./dl.*	23.6	18.7	18.3	
	Lactate / Pyruvate	21.5	17.0	16.8	
24 hrs. urine*	Pyruvate *mg.*	0.46	0.45	0.42	0.41
	α-Ketoglutarate *mg.*	0.69	0.78	0.66	0.63
	Lactate *mg.*	0.72	0.25	0.46	0.59

* The average value of 3 days for each rat.

DISCUSSION

The increase of pyruvate in the blood and urine of thiamine-deficient animals was reported by many investigators, but the increase in α-ketoglutarate in them has not yet been reported, except an observation of Simola (*4*) on the urine of thiamine-deficient rats. The present findings made it clear that α-ketoglutarate in blood increased almost at the same rate as pyruvate, and, moreover, α-ketoglutarate in urine showed an earlier and more marked increase than pyruvate did in thiamine-deficient rats.

As for the riboflavin-deficiency, there were some reports on the increase in pyruvate in blood and urine (*5–7*). In the present study neither pyruvate nor α-ketoglutarate was found to increase, when the other components of the vitamin B group were supplied to the animal in sufficient quantites for complete nutrition, though some increase in them was observed when the dosage of thiamine was of the samllest quantity of the usual range (*2*).

Blood lactate of the thiamine-deficient rats showed some increase, but the degree of the increase was not so marked as that of pyruvate, and thus the ratio of lactate to pyruvate was lowered. This fact has already been observed by several investigators (*8–10*). No tendency toward increase was found in lactate content of the urine and blood of riboflavin-deficient rats.

SUMMARY

1. α-Ketoglutarate as well as pyruvate increased in the urine and blood of thiamine-deficient rats. Especially in the urine the increase of α-ketoglutarate appeared earlier and was more marked than that of pyruvate. Lactate increased somewhat in the blood of thiamine-deficient rats, but the degree of the increase was far lower than that of the increase in pyruvate.

2. Neither pyruvate nor α-ketoglutarate nor lactate was found to increase in the blood and urine of riboflavin-deficient rats as long as the other components of the vitamin B group were given to them in sufficient quantities.

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OXIDATION OF CYANINE AND RELATED DYESTUFFS BY THE ACTION OF LACCASE*

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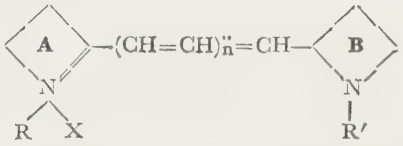
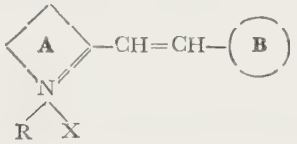
During the past few years a good deal of attention has been directed to the study of physiological and biochemical effects of cyanine, styryl and anil dyes which have long been known as excellent sensitizers for the preparation of panchromatic photographic plates. On the other hand, the study of spectrochemical properties in their relation to molecular structure of these substances has also attracted the interest of physicochemists and organic chemists. Cyanine dyes have the following general structure



n=0, 1, 2, ...

in which **A** and **B** represent heterocyclic nuclei such as quinoline, pyridine, thiazole, *etc.*; R and R′, alkyl groups, and X is acid radical such as halogens. The general formula of styryl and anil dyes are as follows:

Styryl dyes:



where **A**, R, and X represent the same groups as in cyanine dyes, while **B** is a phenyl derivative.

* This work was partly reported before the meeting held by the Botanical Society of Japan and printed briefly in *Bot. Mag.*, **60**, 108, (1947).

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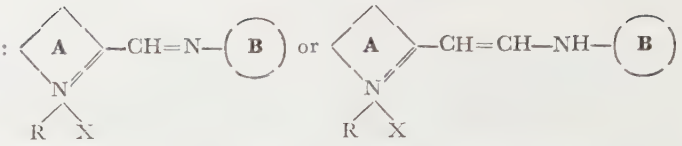
Anil dyes: 

During the course of our study on the effect of these substances upon various enzymes, we found that many of them can readily be oxidized by the action of laccase. In view of the circumstances that a great number of substances with wide varieties of structure were available to us, it seemed worth while to investigate systematically the dependency of oxidizability of each substance upon its structure. The purpose of this paper is to examine this point and clarify the chemical change of the substances caused by the action of the enzyme.

EXPERIMENTAL

The dyes studied were 110 in number, which were kindly supplied us by Dr. T. Ogata of the Scientific Research Institute, Ltd., and by Prof. E. Ochiai of the Phamaceutical Institute, University of Tokyo. Laccase was prepared from a mushroom, *Lactarius piperatus*, according to the method described by Yakushiji (*1*). For comparison, tests were also made with tyrosinase which was isolated from *Lactarius vellereus*. Both these enzymes were stored in saturated ammonium sulfate solution and used after dilution to suitable concentrations.

With most of the dyestuffs the comparative study of the oxidizability by the enzyme was made by measuring the time required for decolorization of the test solutions, since oxidation of the dyestuffs was always accompanied by the disappearance of their original color. For this purpose, dyes were dissolved in 0.01 *M* phosphate buffer at pH 7.0 in a concentration of about 0.001 *M*, and 9 ml. each of these solutions were dispensed in two test tubes, to one of which 1 ml. of the enzyme solution, and to another, 1 ml. of distilled water was added, and the time of decolorization was compared at room temperature (15°).

With some representative cyanine dyes detailed studies were made on the rate of oxidation, the change of absorption spectra in the course of oxidation and also on the nature of reaction products resulting from the enzymatic oxidation. The oxidation process was followed either manometrically using Warburg's respirometer or colorimetrically by measuring the color change with Pulfrich's photometer. In manometric determinations, 2 ml. of dye solutions (0.005–0.0025 *M*) were

mixed with 0.5 ml. of phosphate buffer (pH 7.0) and 0.5 ml. of enzyme solution or distilled water, and the O_2-uptake was measured at 30°. In colorimetric studies, the dyes were dissolved in 0.01–0.001 *M* and the change of optical densities at suitable wave length was followed at 15°. Since aqueous solutions of cyanine dyes did not always follow the Beer's law, the relationship between the photometric reading and dye concentration was previously determined for each dyestuff. Change of absorption spectra accompaning the oxidation process was followed in the range from 430 mμ to 800 mμ at intervals of 10 mμ photoelectrometrically using Adam-Hilger's spectrophotometer. As will be described later in detail, oxidation of cyanine dyes yields several compounds as their end products. In some cases, an attempt was made to separate these products by the chromatographic adsorption method using alumina as an adsorbent. The solution to be separated were previously acidulated with acetate buffer to pH 5.0, and after developing the adsorption column with ethanol or bicarbonate solution the colored portions were cut off and eluted with ethanol or bicarbonate solution. The component thus separated were then subjected to spectrophotometric observations.

RESULTS

Relative Oxidizability and its Relation to the Chemical Structure of the Dyestuffs—The velocity, with which each dyestuff undergoes the action of laccase, was compared by determining the rate of decolorization of test solutions. The results obtained are summarized Tables I–X, in which the following signs are used to indicate the relative velocity of oxidation.

+++: Change of color immediate on the addition of the enzyme.

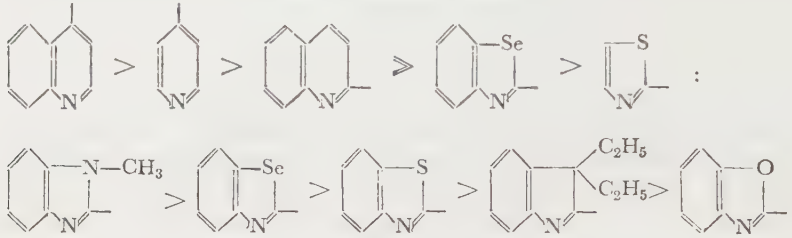
++: Complete change of color occurred 2 hours after the addition.

+: Obvious change of color noticed 24 hours after the addition.

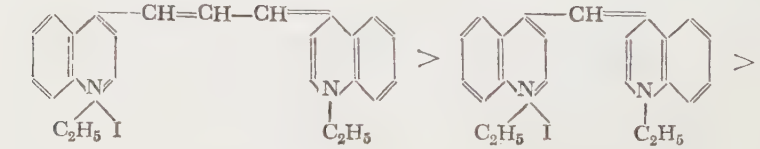
—: No color change observed even 24 hours after the addition.

As will be shown later in detail, certain compounds gave rise to an intermediate which could be reduced to the original substance by $Na_2S_2O_4$. Such cases are indicated in the table by placing the signs in parentheses.

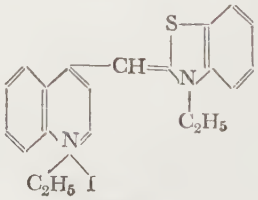
Summarizing the data obtained, we are able to notice certain regularities in the relationship between the oxidizability and the chemical structure of the dyestuffs belonging to each group. For cyanine dyes the following can be stated: (a) The existence of conjugated double bonds between the two terminal nuclei seems to be essential in order that the dyes can be oxidized by laccase (*cf.*, Nos. 31 and 43); (b) The longer the conjugated systems, the higher is the rate of oxidation. (*e.g.*, No. 26 > No. 20; No. 29 > No. 12; No. 23 > No. 11); (c) Among the dyes possessing a conjugated system of the same length, the velocity of oxidation depends on the nature of the heterocyclic nuclei **A** and **B**. In respect to their contribution to the reactivity of the molecule, the nuclei may be placed in the following order:



(d) The acid (X) and alkyl (R) groups attached to the nitrogen atom of the terminal nuclei seem to bear no effect on the oxidizability of the substrate; and (e) In general, there is a tendency that the dyes possessing absorption maxima in a longer wave lengths range are more readily oxidized by laccase. Thus, for example



Absorption maximum: 695 mμ 590 mμ

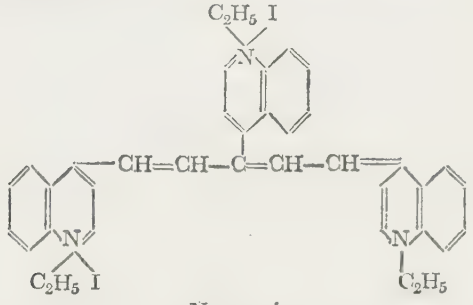


503 mμ

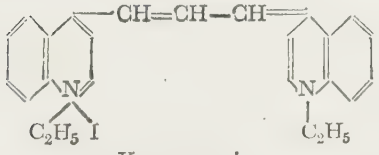
It should be added that no substance belonging to cyanine dyes could be attacked by tyrosinase which is known to be closely related to laccase.

For styryl and anil dyes the following reguralities were brought out on examining the data obtained: (a) At least one hydroxyl or amine group has to be attached to the benzene nucleus B in order that these dyes may be oxidized by laccase; (b) Concerning the influence of radical A, the thesis (c) mentioned above for cyanine dyes also holds true; (c) Also in this case, the acid and alkyl groups attached to the nitrogen atom of the nucleus have no effect on the oxidizability of the substances; and (d) In contrast to the case of cyanine dyes, no distinct relation could be observed between the oxidizability and the position of the absorption maximum.

Details of the Process of Oxidation of Some Cyanine Compounds—(a) Neocyanine and Kryptocyanine.



Neocyanine



Kryptocyanine

These two substances were found to be attacked by laccase in essentially the same way. The aqueous solution of neocyanine is colored green (absorption maxima: 760 and 660 mμ), while that of kryptocyanine is colored blue (absorption maxima: 695 and 620 mμ). The oxidation by the action of laccase took place in two distinct steps: in the first step the color of the dyes completely disappeared, which, however, could be restored if the solution was treated with $Na_2S_2O_4$ before the oxidation proceeded to the second step. Change of the absorption spectrum of neocyanine during the course of the first-step oxidation is

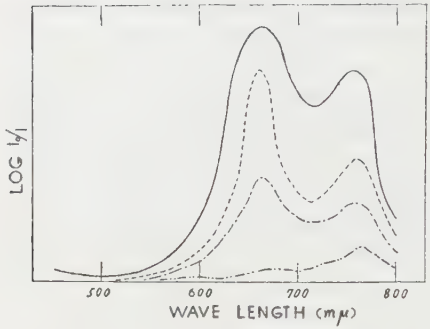


Fig. 1. Change of the absorption spectrum of neocyanine caused by the action of laccase.

———————— : Original spectrum
—·—·—·—·—: 5 minutes after
—··—···—··—: 10 minutes after
— — —·— —: 15 minutes after
the treatment with $Na_2S_2O_4$

illustrated in Fig. 1. On allowing the oxidation to proceed further, the solution assumed a different color which, in the case of neocyanine, was dependent on the experimental temperature. At 30°, an yellow solution was obtained as the final product of oxidation. The color of this solution changed to blue on the addition of $Na_2S_2O_4$. If the solution was kept at 15°, the solution became red which changed to violet by the action of $Na_2S_2O_4$. The absorption spectrum of the final oxidation product, obtained at each temperature, is illustrated in Fig. 2. Such a complicated temperature dependence of the mode of oxidation did not occur in the case of kryptocyanine which yielded a red final solution independent of the temperature (Fig. 3).

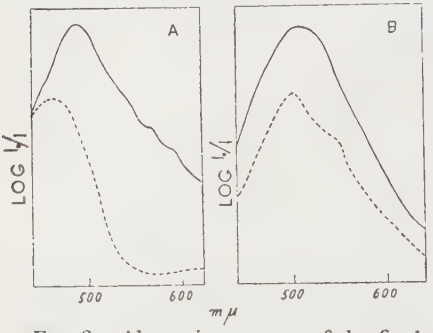


Fig. 2. Absorption spectra of the final oxidation products of neocyanine. (A. at 30°; B. at 15°).

————————: Mixture of oxidation products.
– – – – – –: Main component of the products separated by the chromatogram.

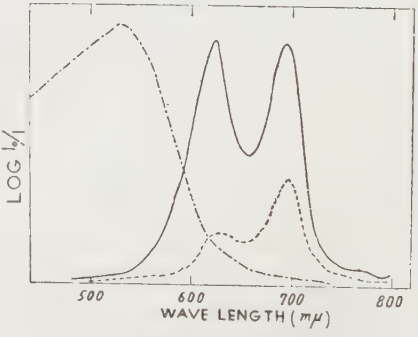


Fig. 3. Change of the absorption spectrum of kryptocyanine caused by the action of laccase.

————————: Original spectrum
— — — —: After 5 minutes
—·—·—·—: After 30 minutes

Manometric measurement of the course of oxygen uptake revealed that in the first reversible step one atom of oxygen was taken up per molecule of the dye, and the completion of the second step of oxidation corresponded to the comsumption of another atom of oxygen. By both manometric and colorimetric measurements it was confirmed that both the first and the second steps of the oxidation represented the first order reaction in respect to the concentration of the substrate (Fig. 4). Note-

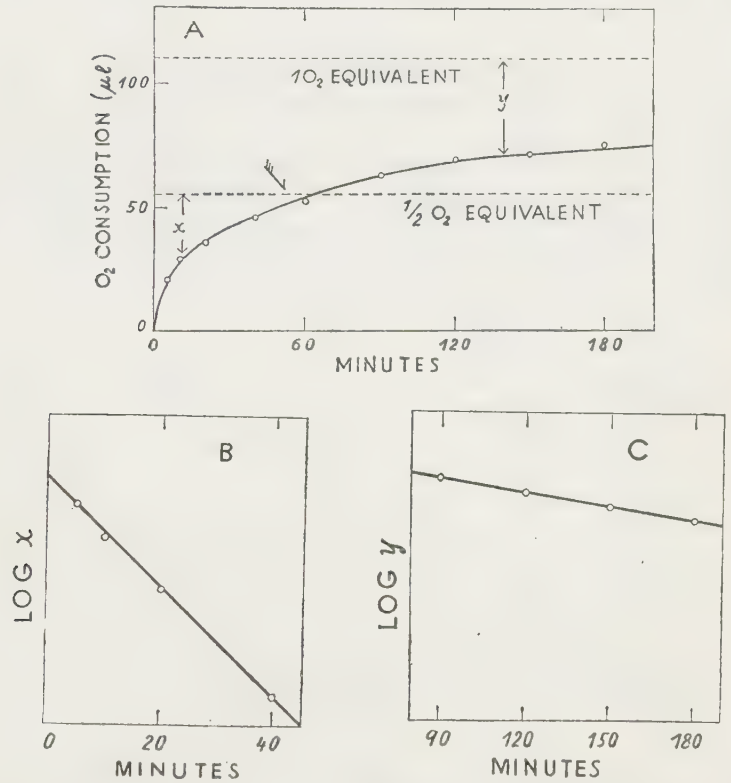


FIG. 4. O_2-absorption by neocyanine in the course of oxidation by laccase. (Neocyanine, 0.0025 *M.*, 2 ml.; buffer, at pH 7.0, 0.5 ml.; laccase, 0.5 ml.; temp., 30°.)

A: The course of O_2-uptake; the arrow indicates the time of complete decolorization.

B: Log x-time relation obtained at the first stage of oxidation.

C: Log y-time relation obtained at the second stage of oxidation.

worthy is the fact that the velocity of the first step oxidation was a function of the laccase concentration, while that of the second step was independent of it, a fact indicating that it was only the first step that was catalyzed by laccase.

Based on these findings it may be concluded that the oxidation of the cyanine dyes studied took place in the following manner:

$$\text{Dyestuff} + \tfrac{1}{2}O_2 \underset{Na_2S_2O_4}{\overset{\text{Laccase}}{\rightleftharpoons}} \text{Colorless intermediate}$$

$$\text{Intermediate} + \tfrac{1}{2}O_2 \xrightarrow{\text{Autoxidation}} \text{End products}$$

the second step occurring in different manners at different temperatures in the case of neocyanine.

The final oxidation products formed were subjected to chromatographic analysis. The results obtained with neocyanine are illustrated in Fig. 5, from which it may be seen that the product formed at 15° was composed of at least two or three components, while that of 30° was composed, at least, of four components. The yellow substance which was common in the two samples (at the top of adsorption column) showed an absorption spectrum as shown in Fig. 2A. On treating with $Na_2S_2O_4$ this component gave rise to a substance showing an absorption spectrum similar to that of diethylcyanine (Fig. 6). Whether this simi-

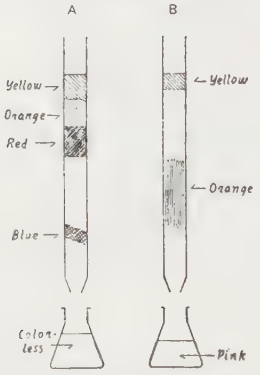


Fig. 5. Chromatogram of the final oxidation products of neocyanine. (A. at 30°; B. at 15°.)

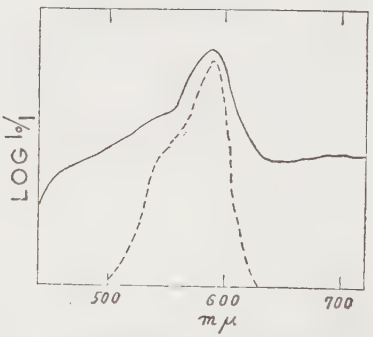


Fig. .6. Absorption spectrum of the reduction product of the yellow component obtained from neocyanine as compared with that of diethylcyanine.

————: Reduction product

— — — —: Diethylcyanine

larity means the identity of the substances, however, cannot be asserted with certainty.

The pink substance which represented the main component of the product obtained at 15° showed an absorption spectrum as illustrated in Fig. 2B. This substance could not be reduced by $Na_2S_2O_4$.

The chromatographic analysis of the end product derived from kryptocyanine showed that it probably contains only one colored substance. This substance, however, was very labile and when eluted from alumina column with alcohol it gave an absorption spectrum as shown in Fig. 7, which might probably be the overlapping of absorption spectra of several substances.

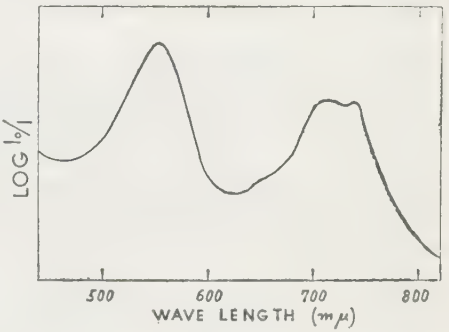
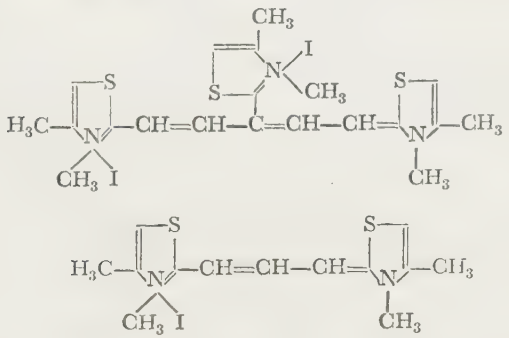


FIG. 7. Absorption spectrum of the final oxidation product of kryptocyanine after elution from alumina column with ethanol.

(*b*) *3-Methylthiazole Derivatives of Neo- and Krypto-cyanines.*



These derivatives are colored violet and red, respectively, in aqueous solutions, and when acted upon by laccase they gave pale yellow solutions, absorbing two atoms of oxygen per molecule (Fig. 8). The intermediate substance which could revert to the original substance by $Na_2S_2O_4$ could not be detected in these cases. It may be reasonable to assume that the oxidation of these substances took place in the same manner as those of neo- and kryptocyanines, the only difference being

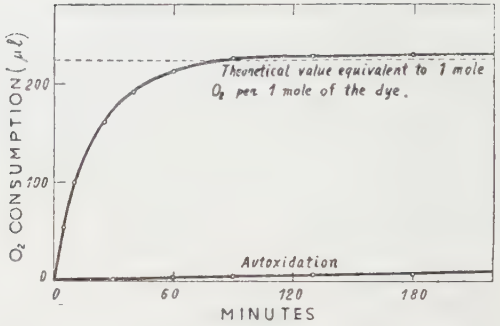
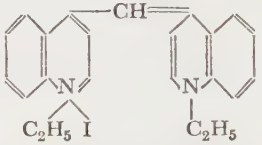


FIG. 8. O_2-absorption by the 3-methylthiazole derivative of neocyanine in the course of oxidation by laccase. (Dye, 0.005 *M.*, 2 ml.; buffer at pH 7.2, 0.5 ml.; laccase, 0.5 ml.; temp., 30°.)

that in these cases the autoxidation of the intermediate formed by the action of laccase proceeded so rapidly that they had escaped our observation.

(*c*) *Diethylcyanine.*



This substance is colored violet in aqueous solution, and when oxidized by laccase it gave pale yellow substance, whereby one atom of oxygen was taken up per molecule of the substance (Fig. 9). This

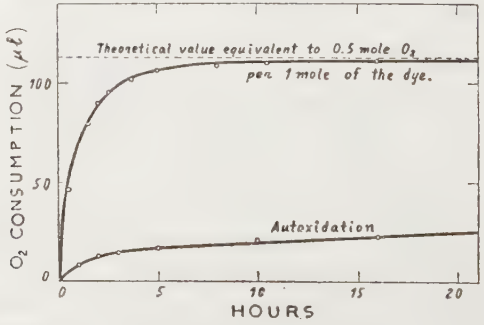


FIG. 9. O_2-absorption by diethylcyanine in the course of oxidation by laccase. (Diethylcyanine, 0.005 *M.*, 2 ml.; buffer at pH 7.0, 0.5 ml.; laccase, 0.5 ml.; temp., 30°.)

product was nonoxidizable, so that the reaction did not proceed further as it was the case with neo- and kryptocyanines. Another difference lies in the fact that the oxidation product of this substance could not be reduced to the original form by the action of $Na_2S_2O_4$.

DISCUSSION

The experiments presented above have shown that a great number of cyanine dyes can be easily oxidized by the action of laccase, while they are refractory to the action of tyrosinase. The chief difference between these two oxidases is already known to lie in the capacity of laccase to oxidize, besides polyphenols and some monophenols, also polyamines such as *p*-phenylenediamine and monomethyl-*p*-phenylenediamine (*2*, *3*) which have two amine groups connected by a conjugated system. The reason why cyanine dyes can be attacked by laccase may reasonably be sought in the fact that they contain two amine groups, one being quartenary and the other tertiary, connected by a conjugated system consisting of methine groups.

It was pointed out that in order for styryl and anil dyes to be affected by laccase, they must contain, besides one tertiary amine group, at least one hydroxyl or amine group in the benzene nucleus in their molecule. This finding corresponds to the fact that aniline, unlike *p*-phenylenediamine or *p*-aminophenol, cannot be oxidized by laccase (*2*).

The salient feature of the enzymatic oxidation of the dyestuffs studied is the disappearance of the strong colors which are characteristic of these dyes. The principal cause of colors of these dyestuffs is known to lie in the existence and the length of the conjugated double bond between the terminal nuclei, though the structure of the nuclei also constitutes an important factor in determining the feature of the absorption spectrum of the dyes. The fact that the oxidation entails the disappearance of the color indicates that the oxidation provokes either saturation or rupture of the conjugated system. In view of the fact, however, that the primary oxidation products could readily be reconverted into the original colored substance by the action of $Na_2S_2O_4$, the possibility of the rupture of the chain may be regarded as quite slight, though it may very probably occur in later, non-enzymatic stage of the oxidation.

Of interest from the theoretical point of view is the fact that there is a certain parallelism between the oxidizability of cyanine dyes and the wave-lengths of their absorption maxima. The explanation of how

this parallelism is established can only come by a fuller understanding of the electronic states of the substances as well as the detail of the action mechanism of the enzyme. At present, all we can say is that the light absorption and the oxidizability of these dyestuffs may probably be conditioned by the same structual factors of their molecules.

SUMMARY

1. Using more than one hundred compounds of cyanine, styryl, anil and related dyestuffs, it was shown that many of them are oxidized to colorless substances by the action of laccase obtained from a mushroom, *Laclarius piperatus*. There is a distinct correlation between the velocity of oxidation and the chemical structure of these dyes.

2. The essential factors determining the rate of oxidation of cyanine dyes were found to be the nature of the two heterocyclic nuclei, the existence and the length of the conjugated double bond between the two nitrogen atoms contained in the nuclei. The cyanine dyes having their absorption maxima at higher wave-length are more readily oxidized by laccase.

3. All cyanine dyes investigated were found to be refractory to the action of tyrosinase.

4. In styryl and anil dyes, the factors determining the velocity of oxidation are the nature of heterocyclic nuclei and the existence of at least one hydroxyl or amine group attached to the benzene nuclei.

5. For some cyanine dyes, the mechanism of oxidation caused by laccase was investigated in detail. It was found that neocyanine and kryptocyanine are oxidized in the following manner:

$$\text{Neocyanine} + \tfrac{1}{2}O_2 \underset{Na_2S_2O_4}{\overset{\text{Laccase}}{\rightleftarrows}} \text{Colorless intermediate}$$

$$\text{Intermediate} + \tfrac{1}{2}O_2 \xrightarrow{\text{Autoxidation}} \text{End products.}$$

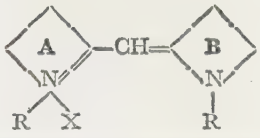
In the case of 3-methylthiazole derivatives of neo- and krypto-cyanines, the secondary autoxidation process occurred so rapidly that the colorless intermediate did not accumulate during the action of the enzyme. In the case of diethylcyanine, the intermediate formed by the action of the enzyme was non-autoxidizable; neither was it reduced to the original substance by $Na_2S_2O_4$.

6. It was discussed that the primary action of laccase upon the

dyestuff may be a certain change in the molecule entailing the disappearance of the system of conjugated double bonds.

In conclusion the authors acknowledge their indebtedness to Prof. H. Tamiya, Dr. Y. Matsuzaki and Mr. K. Shibata for their constant encouragement and helpful suggestions throughout the course of this work. Thanks are also due to Prof. E. Ochiai of the Pharmaceutical Institute, University of Tokyo, and to Dr. T. Ogata of the Scientific Research Institute, Ltd., who kindly placed the invaluable specimens of the dyestuffs at the authors' disposal. This work was supported in part by the Grant in Aid for Fundamental Scientific Research from the Ministry of Education to which the authors extend our grateful thanks.

TABLE I



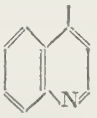
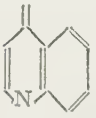
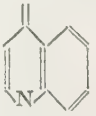
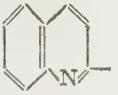
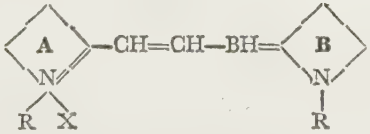
No..	R	X	R	A	B	Velocity of oxidation
1	$-C_2H_5$	—I	$-C_2H_5$			(++)
2	↓ O	$-HClO_4$	$-CH_3$			+
3	$-C_2H_5$	—I	—I			—

TABLE II



* In these tables the following symbols are used: —Me for $-CH_3$; —Et for $-C_2H_5$; —Ac for $-CH_3CO$ and $-\phi$ for $-C_6H_6$.

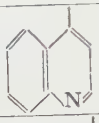
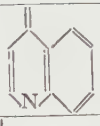
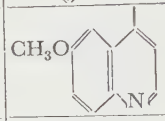
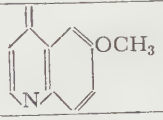
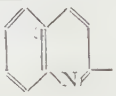
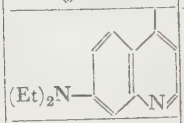
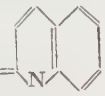
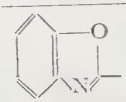
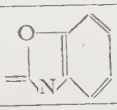
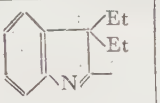
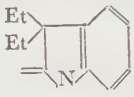
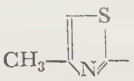
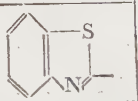
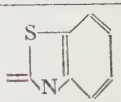
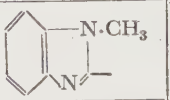
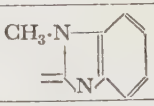
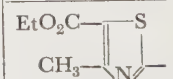
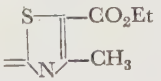
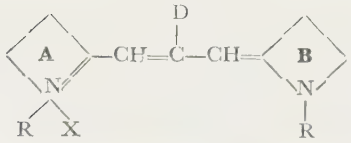
No.	R	X	A	B	Velocity of oxidation
4	$-C_2H_5$	—I			(+++)
5	$-C_2H_5$	—I			(+++)
6	$-CH_3$	—I			++
7	$-C_2H_5$	—I			++
8	$-CH_3$	—I			++
9	$-CH_3$	—I			—
10	$-C_2H_5$	—I			—
11	$-C_2H_5$	—I			++
12	$-C_2H_5$	—I			—
13	$-CH_3$	—I			+++
14	$-C_2H_5$	—I			—

TABLE III



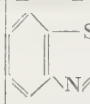
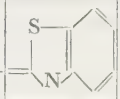
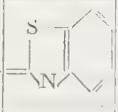
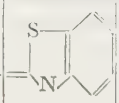
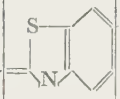
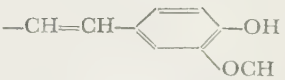
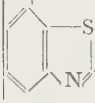
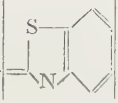
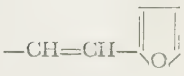
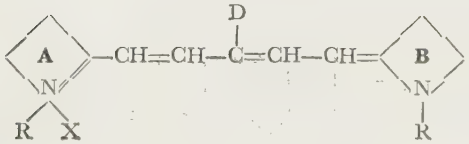
No.	R	X	A	B	D	Velocity of oxidation
15	$-C_2H_5$	—I			$-CH_3$	+
16	$-C_2H_5$	—I			—CH=CH—ϕ	++
17	$-C_2H_5$	—I			—CH=CH—ϕ—N(Et)	++
18	$-C_2H_5$	—I				++
19	$-C_2H_5$	—I				++

TABLE IV



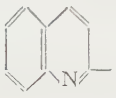
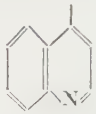
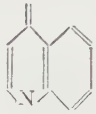
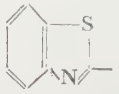
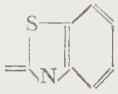
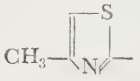
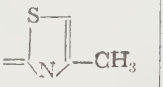
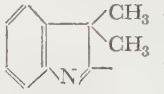
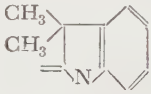
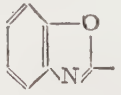
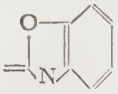
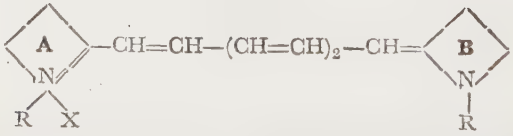
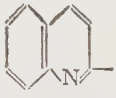
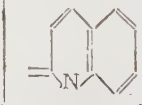
No.	R	X	A	B	D	Velocity of oxidation
20	$-C_2H_5$	—I			—H	+++
21	$-C_2H_5$	—I			—Cl	(+++)
22	$-C_2H_5$	—I			—Cl	(+++)
23	$-CH_3$	—I			—H	++
24	$-CH_3$	—I			—H	+
25	$-CH_3$	—I			—Cl	+

TABLE V



No.	R	X	A	B	Velocity of oxidation
26	$-C_2H_5$	—I			(+++)

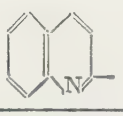
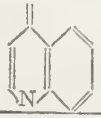
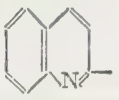
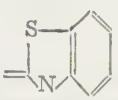
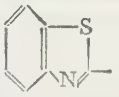
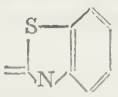
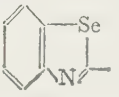
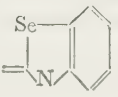
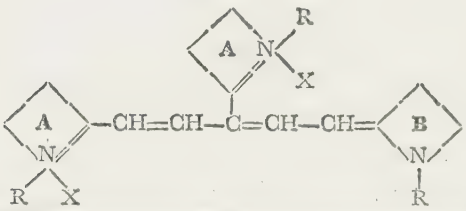
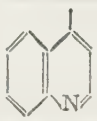
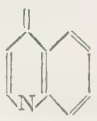
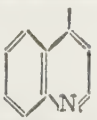
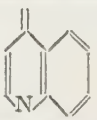
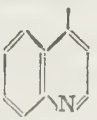
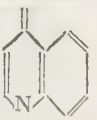
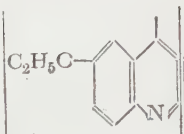
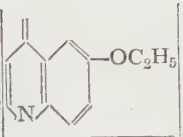
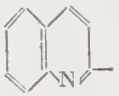
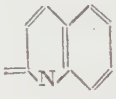
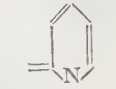
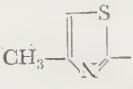
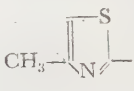
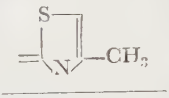
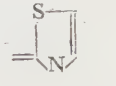
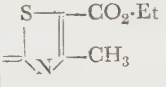
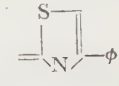
27	$-C_2H_5$	—I			(+++)
28	$-C_2H_5$	—I			(++++)
29	$-C_2H_5$	—I			++
30	$-C_2H_5$	—I			+++

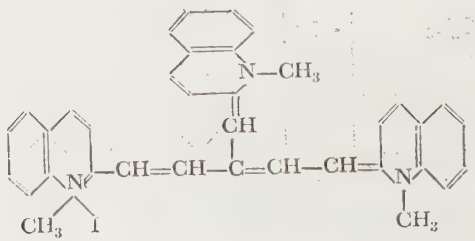
TABLE VI



No.	R	X	A	B	Velocity of oxidation
31	$-C_2H_5$	—I			(+++)
32	$-C_2H_5$	—Br			(+++)
33	$-CH_2-CH_2-OAc$	—I			(+++)

34	$-C_2H_5$	$-I$			(+++)
35	$-C_2H_5$	$-I$			(++)
36	$-CH_3$	$-I$			(++)
37	$-CH_3$	$-I$			+
38	$-CH_2-\phi$	$-I$			+
39	$-CH_3$	$-I$			+
40	$-CH_3$	$-I$			++
41	$-CH_3$	$-I$			++

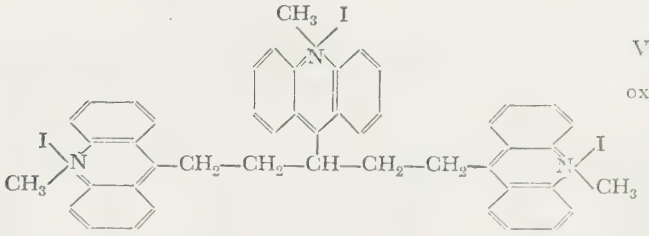
No. 42



Velocity of oxidation

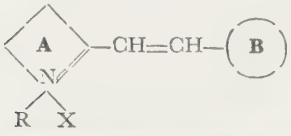
++

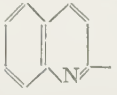
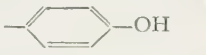
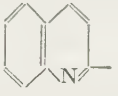
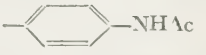
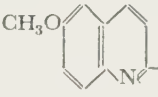
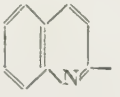
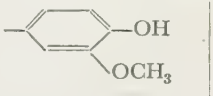
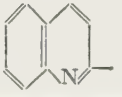
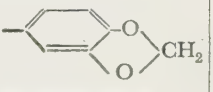
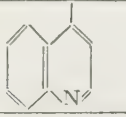
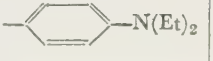
No. 43



Velocity of oxidation: −

TABLE VII



No.	R	X	A	B	Velocity of oxidation
44	$-C_2H_5$	—I			+
45	$-C_2H_5$	—I			−
46	$-C_2H_5$	—I			−
47	$-C_2H_5$	—I			+++
48	$-C_2H_5$	—I			−
49	$-C_2H_5$	—Br			+

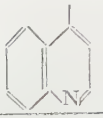
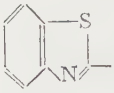
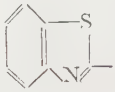
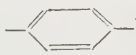
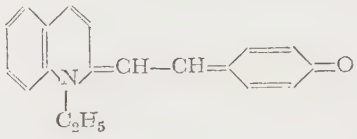
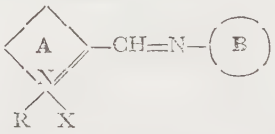
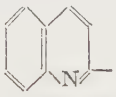
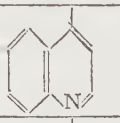
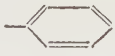
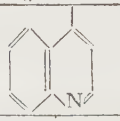
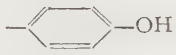
50	$-C_2H_5$	—Br			+++
51	$-C_2H_5$	—Br			+
52	$-C_2H_5$	—Br			—
53	$-C_2H_5$	—Br			—
54					+++

TABLE VIII



No.	R	X	A	B	Velocity of oxidation
55	$-CH_3$	—I			+
56	$-C_8H_{19}$	—I			—
57	$-C_2H_5$	—I			++

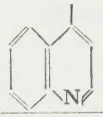
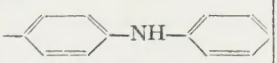
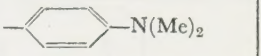
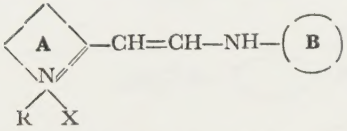
58	$-C_2H_5$	—I			+
59	$-C_2H_5$	—I			+

TABLE IX



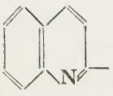
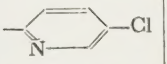
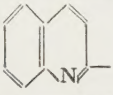
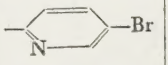
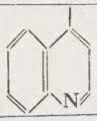
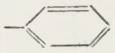
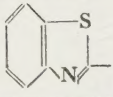
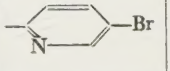
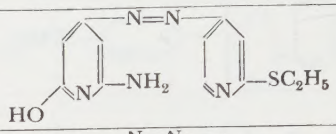
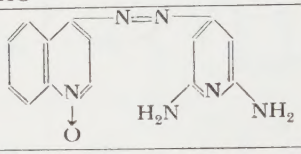
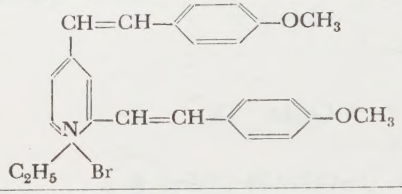
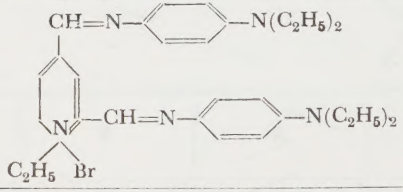
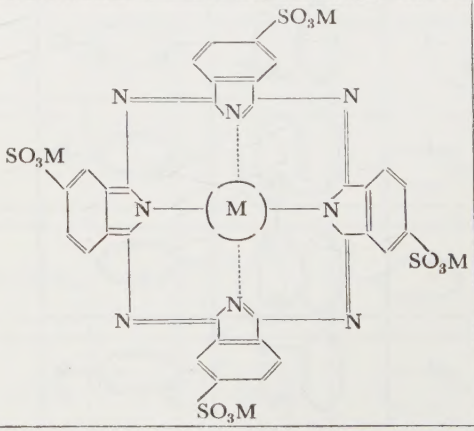
No.	R	X	A	B	Velocity of oxidation
60	$-C_2H_5$	—I			++
61	$-C_2H_5$	—I			+
62	$-C_2H_5$	—I			−
63	$-C_2H_5$	—I			−

TABLE X

No.	Chemical structure		Velocity of oxidation
64			+
65			−
66			+
67			−
68		(M) = Cu, M = Na	−
69		(M) = Cu, M = K	−
70		(M) = Fe, M = Na	−
71		(M) = VO, M = Na	−

REFERENCES

(*1*) Yakushiji, E., *Acta Phytochim.*, **12**, 227 (1941)
(*2*) Yakushiji, E., *Acta Phytochim.*, **10**, 63 (1937)
(*3*) Keilin, D., and Mann, T., *Nature*, **143**, 23 (1939)